

Screening and Examining “Exotic” Chemicals in Swift Current Creek, SK, Canada

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ABSTRACT

A wastewater treatment plant (WWTP) is situated on Swift Current Creek, SK. Although the plant carries out complete secondary treatment followed by UV disinfection, a suite of chemicals is still released into the receiving water. In addition, the City of Swift Current used lagoon treatment for 27 years prior to the construction of the WWTP and thus chemicals in the lagoon could also pose a potential threat to nearby ecosystems. Swift Current Creek flows into Lake Diefenbaker on the South Saskatchewan River system, which is a major drinking water source for Saskatchewan residents. Therefore, the aim of this study was to construct a contaminant chemical profile of the WWTP wastewater and the receiving water throughout the year to monitor the release and fate of pollutants.

Chemical contaminants of interest included hormones, insecticides/pesticides, industrial chemicals and pharmaceuticals and personal care products. A total of 9 sites upstream, downstream and on-site of the WWTP, including the lagoon, were sampled once or twice a month in 2011 and 2012. Organic compounds were extracted via solid-phase extraction using HLB cartridges for effect-directed analysis. Recombinant H4IIE-*luc*, MVLN and MDA cell lines were used to detect dioxin- or PAH-like, estrogenic and androgenic activities, respectively. An HPLC Quadrupole-Orbitrap Mass Spectrometer was used to detect and semi-quantify target chemicals by either full scan or by selected ion monitoring. A mixture of twenty-two chemicals representing each group of compounds was used as a standard for the analysis.

No dioxin- or PAH-like activities were detected using the H4IIE-*luc* bioassay. Significant androgenic activities were detected in influents as expected. However, post-treatment effluent samples illustrated near 100% removal of androgenicity. No estrogenic activities could be detected in any samples, probably due to background levels of hormones found in the growth

media that compromised the sensitivity of MVLN assay. Therefore, Orbitrap Hi-Res coupled to HPLC was used for instrumental analysis to semi-quantify natural and synthetic estrogen mimics. Estrogen equivalency (EEQ) was calculated from instrument analysis results. Seasonal variations were observed as March samples had the highest concentrations of EEQ. The March effluent sample had an EEQ of 2.26 ng/L compared to <0.5 ng/L from the other three sample periods. Longitudinal variations were also apparent, suggesting that input sources other than the WWTP may be present.

Four selected sampling dates representing different seasons of the year (August for late summer, September for early fall, January for middle of winter, and March for early spring) were also analyzed using the Orbitrap. Pesticides were found at greater concentrations in surface water than in raw and treated wastewater. Evidence suggested a major source of input of pesticides to be agricultural leaching and runoff rather than municipal wastewater. Hormones were efficiently removed through the wastewater treatment process as only low levels were detected (mean < 1 ng/L for effluents and downstream surface water). PPCPs exhibited more variable characteristics, as some chemicals had near 100% removal (caffeine and DEET) while others were rather persistent (carbamazepine). Residual concentrations found in effluents and downstream surface waters were in a much wider spectrum, with maximum concentrations of up to hundreds of ng/L. Seasonal variations were observed among PPCPs as well. Concentrations of triclosan and trimethoprim were especially higher in March. Seasons that exhibited elevated chemical concentrations coincided with the spawning seasons of aquatic organisms and thus environmental impacts should be further assessed.

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TABLE OF CONTENTS

PERMISSION TO USE.....	i
ABSTRACT.....	ii
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	v
LIST OF FIGURES	viii
LIST OF TABLES	x
LIST OF ABBREVIATIONS	xi
PREFACE.....	xii
 CHAPTER 1: General Introduction	 1
1.1 Introduction.....	1
1.1.1 South Saskatchewan River Basin and Lake Diefenbaker	3
1.1.2 Swift Current Wastewater Treatment Plant	5
1.2 Chemicals of Concern.....	5
1.2.1 Agricultural Chemicals	5
1.2.1.1 Aquatic Environment Occurrence.....	5
1.2.1.2 Potential Risks and Concerns.....	6
1.2.2 Pharmaceuticals and Personal Care Products (PPCPs).....	8
1.2.2.1 Aquatic Environment Occurrence.....	8
1.2.2.2 Potential Risks and Concerns.....	9
1.2.3 Hormones	12
1.2.3.1 Aquatic Environment Occurrence.....	12
1.2.3.2 Potential Risks and Concerns.....	13
1.3 Analysis Approach.....	15
1.3.1 Effect-Directed Analysis.....	15
1.3.1.1 H4IIE- <i>luc</i> Assay.....	18
1.3.1.2 MDA-kb2 Assay	18
1.3.1.3 MVLN- <i>luc</i> Assay	19
1.3.2 Analytical Chemistry	19
1.3.2.1 Orbitrap	19

1.4 Study Objectives and Hypotheses	20
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CHAPTER 2: Effect-Directed Analysis (EDA) of Waste and Surface Water Samples from a Northern Prairie River

2.1 Abstract.....	24
2.2 Introduction.....	24
2.3 Materials and Methods.....	28
2.3.1 Swift Current Wastewater Treatment Plant	28
2.3.2 Sampling Location and Periods	28
2.3.3 Sample Preparation	29
2.3.4 Effect-Directed Analysis.....	30
2.3.4.1 H4IIE-luc Assay.....	30
2.3.4.2 MDA-kb2 Assay	31
2.3.4.3 MVLN-luc Assay.....	32
2.3.4.4 Statistical Analysis.....	32
2.3.5 Orbitrap Chemical Analysis.....	33
2.4 Results and Discussion.....	34
2.4.1 Dioxin-Like Activities	34
2.4.2 Androgenic Activities	35
2.4.3 Estrogenic Activities.....	37
2.4.4 Orbitrap Results	38
2.5 Conclusions.....	41
2.6 Tables and Figures.....	43

CHAPTER 3: The Fate of “Exotic” Chemicals from Wastewater Effluent Released to a Small Northern Prairie River

3.1 Abstract.....	50
3.2 Introduction.....	51
3.3 Materials and Methods.....	53
3.3.1 Sampling Location and Periods	53
3.3.2 Sample Extraction.....	53

3.3.3 Chemical Standards	54
3.3.4 Orbitrap Chemical Analysis.....	54
3.3.5 Spike Recovery Experiments.....	55
3.3.6 Statistical Analysis.....	56
3.4 Results and Discussion.....	56
3.4.1 Spike Recovery	56
3.4.2 Pesticides found in Swift Current Creek.....	57
3.4.3 PPCPs found in Swift Current Creek.....	58
3.4.4 Hormones found in Swift Current Creek	60
3.4.5 Seasonal Variations in Swift Current Creek	62
3.5 Conclusions.....	64
3.6 Tables and Figures.....	66
 CHAPTER 4: Conclusions.....	 74
4.1 Swift Current Creek	74
4.2 Effect-Directed Analysis and Future Research	78
4.3 Wastewater Monitoring Program	81
4.4 Conclusions.....	83
References	85

LIST OF FIGURES

Figure 1-1: Geological location of Lake Diefenbaker in Saskatchewan. Each yellow pin represents the approximate geological location of major cities in Saskatchewan. City of Swift Current is located upstream of Lake Diefenbaker. The Swift Current Wastewater Treatment Plant releases treated effluent into Swift Current Creek that flows into Lake Diefenbaker. Surface water diverges into South Saskatchewan River, which flows through Saskatoon, and Qu'Appelle River, which flows through Regina.	4
Figure 1-2: Location of Swift Current Creek Sampling Sites.....	22
Figure 2-1: Dioxin-like activities detected in Swift Current Creek in July 2011, August 2011, and January 2012. Each column represents the mean fold change in each sampling site from all three sampling dates. No significant difference can be observed from different sampling dates as well as between different sampling sites.	45
Figure 2-2a: Androgenic activities detected in Swift Current Creek using MDA cell bioassay. Each column represents the mean fold change in each sampling site from all tested sampling dates. Influent samples shown significantly elevated responses (one-way ANOVA, $p < 0.005$), which is expected. Other sampled sites did not elicit any significant responses.....	45
Figure 2-2b: Androgenic activities found in influent samples during different seasons. Seasonal variations are evident, with cellular responses from different sampling dates significantly different from each other (one-way ANOVA, $p < 0.005$).	46
Figure 2-3a: Estrogenic activities detected in Swift Current Creek. Each column represents the mean fold change in each sampling site from all tested sampling dates. Influent samples shown significantly reduced responses (one-way ANOVA, $p < 0.005$), possibly due to cell stress/death due to cytotoxicity. Lagoon samples showed significant, although slightly, elevated responses (one-way ANOVA, $p < 0.005$).....	46
Figure 2-3b: Estrogenic activities found in lagoon samples during different seasons. Lagoon samples had significant estrogenicity detected, with the March sample having significantly higher cellular responses than other sampling dates (one-way ANOVA, $p < 0.005$).....	47
Figure 2-4: Standard curve of MVLN cell bioassay dosed with Estradiol. Significant elicitation above baseline started at approximately 0.046 nM, which is around 12.5 ng/L (one-way ANOVA, $p < 0.005$). High baseline is likely due to high background level of estradiol present in DCC-FBS. Therefore, DCC-FBS has great influence on the sensitivity of MVLN cell bioassays.	47
Figure 2-5: Estradiol equivalencies (EEQ) found in Swift Current Creek from the four sampling dates tested with the MVLN cell bioassay. Estradiol equivalency in this study is the sum of concentrations of E2, E1 and EE2 adjusted with Estradiol Equivalency Factors.....	48
Figure 3-1: Atrazine found in Swift Current Creek downstream of the WWTP. No significant trend to suggest dilution effect, thus it was believed that atrazine was input consistently into Swift Current Creek via non-point sources.....	70

Figure 3-2: Average concentrations of estrogenic hormones detected in Swift Current Creek downstream of the WWTP from four sampling dates. Consistently low levels found in all sites with no trend suggesting dilution effects. Results indicating that low level of point discharge from the WWTP is rather persistent in surface water.70

Figure 3-3: Average concentrations of estrogenic hormones in all downstream sites in Swift Current Creek. As shown, January and March, representing winter and spring (low flow seasons) had higher concentrations than August and September, representing summer and fall season (high flow seasons).71

Figure 3-4a: Seasonal variation of Caffeine and DEET detected in Swift Current Creek. Concentrations of these two chemicals are rather consistent throughout the year due to great removal efficiencies by the WWTP and resulted in relatively low concentrations found in surface water.72

Figure 3-4b: Seasonal variation of Triclosan and Trimethoprim detected in Swift Current Creek. Peak concentration was observed in March which believed to be due to sequestered chemicals in ice released during snowmelt.73

LIST OF TABLES

Table 2-1: Dioxin-like activities detected in Swift Current Creek using H4IIE cell bioassay. Mean fold change value is calculated from replicates of all three sampling dates (July 2011, August 2011 and January 2012). Final concentration of samples in each incubation well is 20X concentrated.	43
Table 2-2: Androgenic activities detected in Swift Current Creek using MDA cell bioassay. Mean fold change value is calculated from replicates of all tested sampling sites. Final concentration of samples in each incubation well is 20X concentrated.	43
Table 2-3: Estrogenic activities detected in Swift Current Creek using MVLN cell bioassay. Mean fold change value is calculated from replicates of all tested sampling sites. Final concentration of samples in each incubation well is 10X concentrated.	44
Table 2-4: Estradiol equivalence found in Swift Current Creek. Estradiol equivalence is the sum of concentrations of E2, E1 and EE2 analyzed by Orbitrap, adjusted by the estradiol equivalence factor determined by MVLN cell bioassay ($E1 = 0.01$, $EE2 = 1.25$, $E2 = 1$). Influent samples had poor recovery due to highly complex matrix. Although calculated concentrations in influent samples do not reflect true concentrations, the poor recoveries help explain cell toxicity and/or nullifying effects observed in MVLN bioassay.	44
Table 3-1: Chemical standard used for Orbitrap analysis and sample spikes.....	66
Table 3-2: Extraction recovery (%) of spiked water samples from three matrices of concern: wastewater influent, wastewater effluent and river water. Each matrix was spiked in triplicate, average results are shown ($n=3$)	67
Table 3-3: Pesticides detected in Swift Current WWTP and Swift Current Creek. Mean values were calculated from all four sampling dates. Non-detect samples are listed as less than detection limit.	67
Table 3-4: Pharmaceuticals and Personal Care Products Detected in Swift Current WWTP and Swift Current Creek. Mean values were calculated from all four sampling dates. Non-detect samples were listed as less than detection limit. Extraction recovery shown in Table 2 were not taken into account.	68
Table 3-5: Natural and Synthetic hormones detected in Swift Current WWTP and Swift Current Creek. Means were calculated from all four sampling dates. Non-detect samples were listed as less than detection limit. EE2, E2 and estrone, despite having low detection limits, have poor extraction recovery in complex matrices (wastewater influents and effluents). Thus values reported are considered to be lowest possible concentrations.	69

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
DEET	N,N-Diethyl-3-methylbenzamide
E1	Estrone
E2	17 β -Estradiol
EDA	Effect-Directed Analysis
EDC	Endocrine Disrupting Chemicals
EE2	17 α -Ethinylestradiol
EEQ	17 β -Estradiol Equivalence
HLB	Hydrphilic-Lipophilic-Balanced
HPLC	High-Performance Liquid Chromatography
MXR	Multixenobiotic Resistance
PAH	Polynuclear Aromatic Hydrocarbons
PPCP	Pharmaceuticals and Personal Care Products
SIM	Selected Ion Monitoring
WWTP	Wastewater Treatment Plant

PREFACE

This thesis has been organized as a manuscript thesis. Therefore, there will be some redundancy of information among chapters. Chapter 1 is a literature review and general introduction to the research. Chapter 4 will provide an overall discussion and conclusion of this thesis, as well as recommendations for future research. Chapters 2 and 3 are in the process of being submitted to peer-reviewed scientific journals for publication. To avoid redundancies in citation lists, all citations have been provided in a combined section at the end of this thesis.

CHAPTER 1: General Introduction

1.1 Introduction

Exotic chemicals released into receiving surface waters have always been a concern. Chemicals from external sources have the potential to disrupt natural habitat, alter aquatic organisms' physiological processes, and eventually may lead to human exposure and potential health impacts. For example, pesticides, which are very abundantly used and present in the prairies, are input into nearby waterbodies via leaching and runoff and have a potential to affect non-target aquatic organisms. Studies have shown that many pesticides can inhibit organisms' multixenobiotics resistance (MXR), which is their first line defence against toxic chemicals (Smital et al, 2004). Herbicides that are flushed into receiving surface waters can also affect algal species (Peterson et al, 2004; Tang and Eschier, 2014). Furthermore, many pesticides are also associated with human health concerns and can raise concerns when detected in drinking water sources (McDuffie et al, 1990; Hoppin et al, 2007; Munger et al, 1997).

In addition to pesticides, endocrine disruptors have been major chemicals of concern for the past 40 years, especially when investigating effects of wastewater in nearby aquatic ecosystems. Endocrine disruptors are foreign chemicals introduced into an organism and can affect the endocrine system in four ways: 1) mimic the effects of endogenous hormones; 2) antagonize the effects of endogenous hormone; 3) disrupt the synthesis of endogenous hormones; and 4) disrupt the synthesis of hormone receptors (Sonnenschein and Soto, 1998). Documented endocrine disruptors included some pesticides, natural and synthetic hormones, plasticizers, detergents, surfactants and more (Sonnenschein and Soto, 1998). Although refuted by many, estrogenic compounds have been linked to adverse effects observed on fish and other aquatic species (Purdom et al, 1994; Jobling et al, 1998; Allen et al, 1999). Estrogen agonists are

suspected to cause demasculinization or feminization in male fish resulting in vitellogenin production, and can potentially affect fish reproduction and growth (Johnson and Sumpter, 2001; Segner et al, 2003). Other endocrine disruptors in the aquatic environment listed by Sumpter (2005) also included androgen agonists/antagonists (masculinization of vertebrates), progesterone agonists/antagonists (behavioural changes), and thyroid antagonists (compromise growth and development) (Sumpter, 2005).

In addition to natural hormones, synthetic chemicals categorized as Pharmaceuticals and Personal Care Products (PPCPs) can also have endocrine disrupting potentials (Daughton and Ternes, 1999). Furthermore, due to the wide range of chemical properties of PPCPs, a variety of other adverse effects may also include MXR inhibition, induction of antibiotic resistance in pathogens, chronic toxicities in aquatic organisms and general bioaccumulation in tissues (Smital et al, 2004; Berglund et al 2014; Quinn et al, 2008; Du et al, 2015). To add to the complexity of the issue, effects of many PPCPs on the aquatic environment still remain unknown.

Surface waters have long been used as repositories for effluents from domestic sewage and agricultural activities. Endocrine disrupting chemicals, including natural hormones and PPCPs, are mainly released into surface water from municipal sewage discharges. Many studies had shown that these chemicals were not completely removed during wastewater treatment (Johnson and Sumpter, 2001; Clara et al, 2005; Nakada et al, 2007). In addition, agricultural and urban runoffs, concentrated animal feeding operations and landfill leachates can also contribute to release of these chemicals as well as pesticides and herbicides to the environment (Benotti et al, 2009). More importantly, surface waters also serve as drinking water sources for both humans and wildlife. Studies have detected chemical contaminants in drinking water reservoirs as well as in treated drinking water (Donald et al, 2007; Benotti et al, 2009). Therefore, the occurrence and

behaviour of trace chemicals needs to be closely examined in wastewater effluents and receiving water bodies.

1.1.1 South Saskatchewan River Basin and Lake Diefenbaker

The South Saskatchewan River is 1,392 km long and rises in southern Alberta and flows east. It supports a population of approximately 3 million people as a major water source for Alberta, Saskatchewan and Manitoba (North et al, 2014). From Alberta the river flows east, entering and passing through Saskatchewan, and empties into Lake Winnipeg in Manitoba. Approximately 45% of the population of Saskatchewan relies on the South Saskatchewan River for their daily water needs. The South Saskatchewan River receives chemical and waste discharges throughout its course, including from municipal and industrial point source discharges, feed lots, and non-point source agricultural runoff.

Lake Diefenbaker is a man-made lake that was formed by the construction of the Gardner and Qu'Appelle River dams in the 1960s. It is located approximately 100 km southwest of Saskatoon. It is a valuable freshwater reservoir that serves as a source of drinking water for both local and downstream communities (North et al, 2014). The lake diverges into the Qu'Appelle River and South Saskatchewan River. The South Saskatchewan River flows northeast through Saskatoon while the Qu'Appelle River flows southeast. The two rivers serve populations of two major cities in Saskatchewan: Regina and Saskatoon (Lindenschmidt and Sereda, 2014).

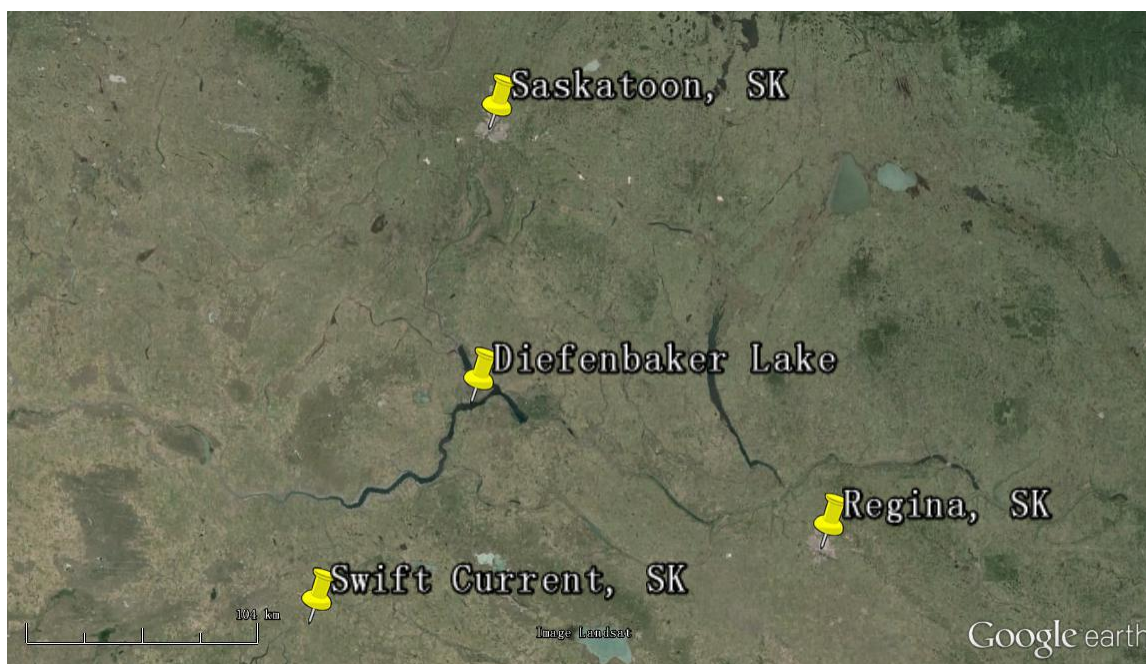


Figure 1-1: Geological location of Lake Diefenbaker in Saskatchewan. Each yellow pin represents the approximate geological location of major cities in Saskatchewan. City of Swift Current is located upstream of Lake Diefenbaker. The Swift Current Wastewater Treatment Plant releases treated effluent into Swift Current Creek that flows into Lake Diefenbaker. Surface water diverges into South Saskatchewan River, which flows through Saskatoon, and Qu'Appelle River, which flows through Regina.

This study aimed to quantify inputs of a variety of chemical compounds to Lake Diefenbaker via Swift Current Creek, a small tributary of the South Saskatchewan River. Swift Current Creek flows through the city of Swift Current, traverses approximately 160 km through the Saskatchewan prairies before it empties into the South Saskatchewan River at Lake Diefenbaker. Along its length Swift Current Creek receives a variety of inputs including agricultural runoffs and most significantly wastewater from the wastewater treatment plant (WWTP) in Swift Current, SK. Wastewater treatment plants are a major source of foreign chemicals in receiving waterbodies. Municipal sewage contains natural and synthetic compounds that can influence aquatic organisms and their hormonal systems (Ternes et al, 1999). In addition, many pharmaceuticals are constantly released into receiving water bodies and may have targeted

and non-targeted effects on the aquatic ecosystem (Daughton and Ternes, 1999). Thus, wastewater effluents introduce a suite of exotic chemicals into Swift Current Creek which eventually flows into Lake Diefenbaker. Therefore, it is important to assess the chemical constituents of the effluents and consequently the effects on aquatic ecosystems in Lake Diefenbaker and the South Saskatchewan River.

1.1.2 Swift Current Wastewater Treatment Plant

The original lagoons that treated wastewater for Swift Current, SK were constructed in 1955. Discharges from these lagoons were made to Swift Current Creek annually during the spring and fall. Due to population increase over the years, the city developed a new wastewater treatment plant in 2006. The WWTP treats 5 million litres of sewage daily for a population of approximately 15,000 people (2011). The plant operates using primary treatment to filter larger solids followed by secondary activated sludge treatment to breakdown organic matter. The remaining solids are removed via sedimentation and the treated effluent goes through UV disinfection before being released to Swift Current Creek.

1.2 Chemicals of Concern

1.2.1 Agricultural Chemicals

1.2.1.1 Aquatic Environment Occurrence

Pesticides are widely applied on the Canadian prairies due to the pre-eminence of the agriculture industry. In 1997, at least 18 million kilograms of pesticide, which was 36% of the 50 million kg Canadian usage, was used in Saskatchewan (Hjertaas, 2007). Also, over 88 and 90 million kg of pesticides were sold in 2010 and 2011, respectively, and approximately 70% of the

total in each year was sold to agricultural sectors (Health Canada, 2011). Continuing use of large quantities of pesticides on the prairies is a cause for environmental and ecological concerns, as these pesticides enter the aquatic ecosystems via agricultural runoff. A study conducted in 2008 detected select pesticides at higher frequencies than pharmaceuticals, plasticizers and flame retardants in 74 sites in the United States, including both surface water and ground water (Focazio et al, 2008). US scientists have detected high concentrations of atrazine, a widely used herbicide that is suspected to be an endocrine disruptor, in the source water of almost all drinking water treatment plants in USA that they have studied (Benotti et al, 2009). More significantly, they found that the highest concentrations of atrazine occurred in source water closest to agricultural lands. Drinking water treatment reduces herbicides detected in a reservoir by an average of 14 – 86% with variation depending on the herbicide (Donald et al, 2007). Nevertheless, many herbicides are still present in treated drinking water at detectable concentrations. In the said study by Donald et al (2007), the water supplies of 15 communities in Manitoba, Saskatchewan, and Alberta were investigated for chemical characterization. Two insecticides, twenty-seven different herbicides and two degradation products were detected in 15 reservoirs at total concentrations ranging from 48 ng/L to 1,062 ng/L.

1.2.1.2 Potential Risks and Concerns

Movement of pesticides from targeted areas to non-target areas may pose potential risks to the environment, as they may have unintended effects on natural inhabitants (i.e. aquatic organisms), and the ecosystem as a whole. A study by Peterson et al (1994) determined the phytotoxicity of 23 different pesticides applied at expected environmental concentrations. Ten algal species and one vascular plant were exposed to these pesticides and they discovered a wide

range of species sensitivity (Peterson et al, 1994). Certain compounds were highly toxic to some species while having no effects on other species. The results revealed further complexity to predicting ecosystem effects, as it suggested the species sensitivity varies significantly, even at the lowest trophic level in aquatic ecosystems. In a study by Smital et al (2004), 15 commonly used pesticides were tested at environmentally relevant concentrations and it was found that zebra mussel exposed to pirimicarb, endosulfan, chlorpyrifos-methyl, malathion, or dichlorvos showed decreased MXR. MXR is the first line of defence in aquatic organisms against endogenous and exogenous toxicants by transporting them out of the cell by various transmembrane transport proteins (Smital et al, 2004). It was determined that compromised MXR is mainly due to bioaccumulation and additive mixture effects of multiple pesticides present in the aquatic environment (Smital et al, 2004). A recent study used a mixture consisting of model herbicides at environmentally realistic concentration to assess herbicide toxicity (Tang and Eschier, 2014). It was concluded that photosystem II herbicides dominate algal toxicity by inhibiting both photosynthesis and growth. An earlier study did a comparative assessment of different available bioassays to assess acute toxicity of carbofuran, cyromazine, fenamiphos, formetanate and propamocarb. This study presented EC_{50} s in the low mg/L range. However, similar to the aforementioned study by Smital et al, mixtures of these pesticides had even lower EC_{50} s, and therefore, increased toxicity (Fernandez-Alba et al, 2001). In addition to ecological issues, a major concern with contamination by pesticides is that they are often associated with human health anomalies. Previous research has also shown that atrazine, metolachlor, and cyanazine when present in drinking water were each significant predictors of intrauterine growth retardation in human births (Munger et al, 1997).

1.2.2 Pharmaceuticals and Personal Care Products (PPCPs)

1.2.2.1 Aquatic Environment Occurrence

One major contaminant group found in effluents are pharmaceuticals and personal care products (PPCPs). Although drugs entering living organisms are subject to metabolism, a significant fraction leaves the human or animal body in their active form. As a result, these drugs are secreted in urine and feces and are eventually emitted into raw sewage. Even though effluents are treated through multiple procedures before being released into the environment, not all chemicals are efficiently removed. A study in 2004 demonstrated that overall removal efficiency of PPCPs by primary and secondary treatment ranged from 60 – 90%, suggesting that significant amounts of chemicals remained in the final effluent (Carballa et al, 2004). To add to the complexity, Clara et al (2005) concluded that some pharmaceuticals pass through treatment plants without any reduction at all, as effluent concentrations were in the same range as influent concentrations. Furthermore, improved treatment technologies did not increase removal efficiency, as comparable removal rates were observed between conventional activated sludge methods and newer membrane bioreactors (Clara et al, 2005). In summary, PPCPs could potentially be present in significant concentrations in treated wastewater effluents.

PPCPs are also considered to be pseudo-persistent, since their constant, relatively great input into the environment often exceeds the rates of transformation and removal (Petrovic et al, 2003). Non-prescription drugs, including caffeine, ibuprofen and acetaminophen, were detected at very high frequency in many surface water and groundwater sites (Focazio et al, 2008). The same study concluded that higher detection frequencies occurred in surface water sites compared to groundwater sites, indicating a more direct source of input of these chemicals, such as wastewater effluents. A study in the UK performed targeted analysis of a suite of

pharmaceuticals (Roberts and Thomas, 2006). Not only did they estimate daily environmental input of 11 pharmaceuticals including diclofenac, clofibric acid, and ibuprofen, they also measured their concentrations in receiving water. There was no correlation between estimated daily input and environmental concentrations in receiving waters, suggesting that some pharmaceuticals, notably ibuprofen, are more persistent than others. Therefore, it is not surprising that many PPCPs are still detected in both drinking water sources and finished drinking waters. One study reported 40 chemicals that were detected in upstream or raw water and 17 that were found in finished drinking water (Stackelberg et al, 2004). A study in California on reclaimed wastewater also detected 14 chemicals, including pharmaceuticals and hormones, in the $\mu\text{g/L}$ range (Soliman et al, 2004). Another study in California revealed seasonal variations in concentrations of PPCPs in wastewater and drinking water (Loraine and Pettigrove, 2006). The variation was mainly due to different flow rates in receiving water bodies throughout the year, as low flow rates lead to higher concentrations of chemicals and vice versa.

1.2.2.2 Potential Risks and Concerns

PPCPs pose potential risks to aquatic wildlife as well as human health based on a variety of modes of action and exposures in receiving surface waters. Firstly, due to the nature of drugs, which target specific biological functions in a variety of species, these chemicals are designed to be highly potent. In addition, since many drugs need to travel around the human body before reaching their site of action, and may be required to exert their effects for extended periods of time, some are designed to be relatively resistant to biodegradation (Ternes and Hirsch, 2000). Lastly, PPCPs are generally constantly released into receiving waters from the effluents and thus have the potential to be present in the aquatic environment at elevated concentrations for

extended periods of time. PPCPs therefore have the potential to persist in the environment and to have adverse chronic effects even at relatively low concentrations (Ternes and Hirsch, 2000).

PPCPs can have targeted or non-targeted effects on aquatic organisms. Some drugs including antibiotics and hormones have direct effects on the aquatic organisms and their ecosystems (Daughton and Ternes, 1999). Hormone mimics could elicit receptor-regulated effects in aquatic organisms, while antibiotics in receiving waters can induce pathogen resistance or affect microbial communities. As mentioned earlier, antibiotics could induce pathogen resistance, and as a result could affect the health of other organisms. For example, a recent European study has shown that wastewater treatment plants and their effluents may contribute to increases in antibiotic resistance in environmental bacteria (Berglund et al, 2014). Berglund et al (2014) observed a significant correlation between the concentration of antibiotics detected in water and concentration of antibiotic resistance genes. The potential implications from this finding are not only disruption of the natural ecosystem, but also possible human health concerns related to the control of bacterial infection due to the development of drug resistance. In the aforementioned study performed by Smital et al (2004), they also discovered MXR inhibitory potential in synthetic musks, which are commonly found in wastewater effluents. By incubating different commercial musks with the test organism California Mussel and indicator chemicals, increase in accumulation of the indicator was observed (Smital et al, 2004). More interestingly, they also stated that the inhibitory effect persisted for 1 – 2 days after exposure. Combine this with the pseudo-persistence characteristic of many PPCPs, it could pose a great concern for receiving ecosystems.

Previous studies mainly focused on acute toxicity of PPCPs on aquatic organisms. However, since PPCPs are continuously present at low concentrations, chronic toxicity tests may

be more appropriate to assess potential effects of PPCPs (Quinn et al, 2008). A study in Montreal, QC used *Hydra attenuata* to assess acute and chronic toxicity of 11 compounds that are frequently detected in wastewater effluents. Based on their results as well as using classifications from EU directive 93/67/EEC, gemfibrozil, ibuprofen and naproxen are considered to be toxic with chronic EC₅₀s between 1 and 10 mg/L, concentrations well above their usual much lower presence in the aquatic environment. Although these levels were generally much higher than concentrations usually found in effluents and receiving waterbodies, many contaminants have the tendency to bioaccumulate into much higher concentrations in organisms of higher trophic levels. Using the same standards, carbamazepine, bezafibrate, sulfapyridine, oxytetracycline and novobiocin are also considered harmful, with their chronic EC₅₀ values in the range of 10 – 100 mg/L (Quinn et al, 2008). It is rather difficult to predict unknown toxicity for many drugs, as different drugs in the same class could have very different toxicities. In addition, many drugs only elicit subtle effects that researchers tend to overlook. These subtle changes however, could greatly impact species populations and the ecosystem as a whole (Daughton and Ternes, 1999).

Finally, in addition to targeted modes of action, PPCPs could also cause non-targeted toxic responses through bioaccumulation. Pharmaceuticals, due to their high bioavailability, tend to bioaccumulate in various aquatic organisms including fish, mussels and algae (Du et al, 2015). Although information on mechanisms of uptake and elimination of many pharmaceuticals are still unknown, their study showed that drugs from many pharmaceutical categories were detected both in surface water and in aquatic organisms (Du et al, 2015). Previous research had revealed that 25 different PPCPs were present in Waskana Creek, which receives municipal wastewater from the City of Regina SK. In addition, these scientists detected concentrations of ibuprofen, naproxen, sulfamethoxazole, gemfibrozil, erythromycin and triclosan that may present a risk to

aquatic organisms (Waiser et al, 2011). As mentioned previously, antibiotics released into sewage effluents are also suspected to induce resistance in bacteria. This presented human health concerns as increasing numbers of infections cannot be cured with antibiotics. Antibiotic-resistant strains of bacteria are found in sewage effluents, receiving water, as well as drinking water (Hirsch et al, 1999).

1.2.3 Hormones

1.2.3.1 Aquatic Environment Occurrence

Natural and artificial hormones released into sewage pose great risks to aquatic organisms and humans. Natural and synthetic hormones such as estrone (E1), 17 β -estradiol (E2), 17 α -ethynylestradiol (EE2) and 16 α -hydroxyestrone are frequently detected in Canadian sewage effluents (Ternes et al, 1999; Carballa et al, 2004). Human excretion of 17 β -estradiol is in the range of 2 – 100 μ g daily and as high as 30 mg/day in pregnant women (Huang and Sedlak, 2001). As a result, high levels of hormones could be present in municipal sewage. Johnson and Sumpter (2001) conducted a critical review summarizing removal efficiency for estrogenic hormones from sewage. They compared different studies and concluded that activated sludge treatment strategy can remove E2 and EE2 at high efficiencies of over 85% while E1 was eliminated at lower and more variable efficiencies. Regardless, residual concentrations of estrogenic hormones are still detectable in wastewater effluents as well as receiving waters due to high concentrations in raw sewage. Several studies had detected ng/L concentrations of both E2 and EE2 in wastewater effluents in different countries (Ternes et al, 1999, Huang and Sedlak, 2001). Surface waters were also shown to have detectable E2 and EE2 concentrations just below 1 ng/L (Huang and Sedlak, 2001). Although detected levels were generally low, it is reasonable

to assume that fluctuations in these concentrations will occur due to variable effluent release, water flow and precipitation. Despite variable low concentrations, naturally produced hormones and steroids were the most frequently detected group of compounds in many surface water sites as well as groundwater sites (Focazio, 2008). The sites in this study were all chosen in areas that were known or suspected to have one or more human and/or animal wastewater sources upstream. Many artificial endocrine disruptors, including bisphenol-A and nonylphenol and its derivatives, were also detected in wastewater influents and effluents in ng – µg/L ranges (Clara et al, 2005). Similar to hormones discussed in the previous section, many of these chemicals are not efficiently removed during the treatment processes, with some chemicals showing no reduction in concentration at all (Clara et al, 2005).

1.2.3.2 Potential Risks and Concerns

Hormones are secreted by organisms to regulate internal homeostasis and to acclimate to environmental factors and changes. Natural and synthetic hormones released from sewage discharges are considered to be a major group of endocrine disruptors (EDCs) as they mimic the effects of native hormones used by aquatic organisms in receiving environments and alter normal reproductive or developmental processes. Endocrine disruptors are foreign chemicals introduced into an organism and affect the endocrine system in four ways: 1) mimic the effects of endogenous hormones; 2) antagonize the effects of endogenous hormone; 3) disrupt the synthesis of endogenous hormones; and 4) disrupt the synthesis of hormone receptors (Sonnenschein and Soto, 1998). The most notable and widely studied type of environmental EDCs are natural and synthetic estrogen receptor agonists. Many alkylphenols as well as bisphenol-A (BPA) are also considered to be xenoestrogens (Sonnenschein and Soto, 1998; Rubin, 2011). The main

correlated adverse effects on aquatic organisms are feminization and/or demasculinization of fish and amphibians. Studies over the years have progressively made scientists believe that estrogenic chemicals found in wastewater effluents can have adverse effects on fish as well as other aquatic organisms (Purdom et al, 1994; Jobling et al, 1998; Allen et al, 1999). In summary, EE2 in wastewater effluents poses the greatest risk of impact to wildlife due to its high potency in affecting sexual differentiation in fish, followed by E1 and E2 (Johnson and Sumpter, 2001). A European project known as “Identification of Endocrine Disrupting Effects in Aquatic Organisms (IDEA)” determined that EE2 was able to alter fertilization success and vitellogenin induction in zebrafish at a concentration as low as 1.67 ng/L (Segner et al, 2003). Furthermore, the same study showed that reproduction in zebrafish was completely inhibited at EE2 concentration of 10 ng/L. Also, this study had determined that aquatic organisms are especially sensitive to estrogenic hormones during sexual differentiation. In a critical review by Sumpter (2005), he summarized that both estrogenic and androgenic EDCs are widely present in the aquatic environment and both masculinization and feminization of fish had been observed and reported. At the currently stage, direct effects of endocrine disrupting chemicals on humans have not been clearly identified. Many publications from the early years have tried to correlate exposure to EDCs and reproductive abnormalities or incidences of cancers observed in humans (Davies et al, 1993; Carlsen et al, 1992; Sharpe and Skakkebaek, 1993). However, further studies have not supported these proposed theories (Safe, 2004). Nonetheless, due to their presence in the aquatic environment as well as drinking water sources, it is important to investigate EDCs and their potential effects in this study.

In addition to well-known estrogen agonists, bisphenol-A is now another major EDC, affecting organisms through multiple regulatory pathways. Although BPA is a weak

environmental xenoestrogen, recent studies have demonstrated that it can still stimulate cellular responses at very low concentrations (Hugo et al, 2008). Studies have also shown that BPA binds strongly to estrogen related receptor gamma, a receptor that is highly expressed in the placenta. Consequently, it could affect fetal development (Takeda et al, 2009). Other mechanisms of action for BPA were summarized by Rubin (2011) and include: interfering with thyroid hormone pathways, acting through the glucocorticoid receptor, and acting as an androgen receptor antagonist (Rubin 2011).

1.3 Analysis Approach

1.3.1 Effect-Directed Analysis

Samples extracted from complex matrices such as wastewater influents and effluents are generally complex mixtures containing large numbers of chemicals. Analyses of these samples are usually limited by the methodology used to analyze the mixture, limited availability and huge costs for the required standards, and the lack of knowledge for the majority of the chemicals (Hecker and Hollert, 2009). Concentrations of each chemical present in the sample could range over several orders of magnitude, which makes the development of methodologies accommodating all compounds nearly impossible (Hecker and Giesy, 2011). In addition, to accurately analyze each compound, relevant standards should also be analyzed, which could quickly make the study very expensive due to the number of chemicals to be analyzed. Finally, the lack of information on the majority of these chemicals makes the sole concentrations of many chemicals irrelevant in terms of environmental impact (Hecker and Giesy, 2011).

To overcome these challenges, effect-directed analysis (EDA) approaches have been developed to address the limitations of sole chemical analysis. EDA typically involves a series of

bioanalytical methods coupled with instrumental analytical methods that can eventually identify causal agents from a chemical mixture and quantify chemicals of interest. These bioanalytical methods usually utilize certain properties of particular chemical groups to elicit biological changes that are proportional to the dose, typically *in vitro* or *in vivo* responses (Hecker and Giesy, 2011). Once positive samples are identified, fractionation is performed to separate the chemical mixture based on different chemical or physical properties. Each fraction is then tested with the same bioanalytical assay and the positive fractions are isolated again. This process is repeated until one or few positive fractions are identified that only contain a few chemicals. The identified fractions are then analyzed by an analytical instrument to qualify and quantify causal agents.

One major advantage of EDA is that the screening methods are quick and cost-effective, require only 1 or 2 standards that represent the typical agonist and(or) antagonist effects of the biological system. In addition, the measurable elicited biological effect is directly proportional to the overall effect of the mixture, accounting for toxic potency of each chemical present as well as interactions among chemicals (Hecker and Giesy, 2011). Thus EDA also provides a more relevant assessment of environmental risks and impacts as chemicals are generally present as a mixture. Lastly, EDA helps account for effects of unidentified chemicals in a mixture, which provides us a more reliable prediction of the environmental impact.

An EDA approach has to be thoroughly planned and applied, as limitations and challenges are also present. Firstly, though EDA accounts for effects of unidentified chemicals, it is often difficult to use EDA to pinpoint causal agents of the biological effects (Sanderson et al, 1996). Secondly, because the effects of many chemicals are unknown, many of these chemical groups lack established EDA methods to assess their biological effect (Hecker and Hollert, 2009).

Sensitivities of bioanalytical methods are generally not as high as instrumental analytical methods. However, highly concentrated samples could result in complex toxic effects to the biological system (Hecker and Hollert, 2009). Lastly, although the chosen biological indicators may be realistic to individual species, they may not reflect the entire spectrum of potential environmental impacts.

The EDA approach applied in this study utilized different cell bioassays, coupled with Orbitrap Hi-Res HPLC analysis to assess toxicity in wastewater as well as receiving surface waters. Cell bioassays are based on cell lines that have specific receptors for specific chemical groups. The receptors control gene expressions, which produce measurable endpoints that are proportional to the concentration of substrates that the cells are exposed to. The cell lines used in the bioassays applied in this study have a recombinant luciferase reporter gene, which produces luminescence that can be detected by our plate reader. H4IIE, MVLN, and MDA cell bioassays were used to assess samples for different effects that are frequently observed in an aquatic environment. The H4IIE-*luc* cell assay detects planar hydrocarbons such as polycyclic aromatic hydrocarbons (PAHs), dioxins/furans, and certain pesticides (Aarts et al, 1995). MVLN and MDA cell assays measure (anti)-estrogen and (anti)-androgen activities respectively (Wilson et al, 2004; Wilson et al, 2002). These assays supplemented my chemical analysis, allowed me to not only construct a chemical profile for influents, effluents and surface waters, but also to assess their endocrine disrupting and other toxic potentials. No further fractionation procedures were performed post instrumental analysis due to required labour and budget constraints.

1.3.1.1 H4IIE-*luc* Assay

The H4IIE-*luc* cell line was used to detect PAHs and dioxin-like chemicals in sample extracts. These types of chemicals exhibit a planar shape and are able to bind to the aryl hydrocarbon (Ah) receptor. Upon binding of the ligand to the receptor, a cascade of regulatory processes is activated and the effect of chemicals is derived. The H4IIE-*luc* cell line is a recombinant cell line that has been created that possesses Ah receptor-controlled expression of a luciferase reporter gene. This recombinant cell line has greater selectivity, sensitivity and dynamic range compare to its wild type (Sanderson et al, 1996). This and other similar cell assay methods compliment analytical instrumental analysis methods as they are less labour intensive and so are less expensive (Aarts et al, 1995).

1.3.1.2 MDA-kb2 Assay

This study also used the MDA-kb2 cell line to detect chemical that agonistically or antagonistically bind to the androgen receptor. This assay utilizes a transformed MDA human breast cancer cell line that contains the androgen-responsive luciferase reporter plasmid to detect androgenic and anti-androgenic activities (Wilson et al, 2002). The cells show response to androgen receptor (AR) agonist Dihydrotestosterone (DHT) at very low concentrations (LOEC = 0.1 nM) and are very sensitive to AR antagonist hydroxyflutamide (OHF) (0.05 μ M) (Wilson et al, 2002). DHT was used as a standard for this assay at concentrations listed in the literature. As stated in the literature, when testing anti-androgenic effects, the AR antagonist OHF combined with DHT is used as a positive control for anti-androgenic effects. In this study, the sensitivity of this assay allowed us to detect as low as 5 pM (0.5 – 1.5 ng/L) of androgenic compounds in surface water. In previous studies, MDA bioassays had demonstrated a wide range of uses in the

detection of androgenic and anti-androgenic effects in cosmetic products (Ma et al, 2003) as well as in the effects of polybrominated diphenyl ethers (PBDE), a widely used commercial product (Stoker et al, 2005).

1.3.1.3 MVLN-luc Assay

Similar to the MDA-kb2 cell line, the MVLN cell line was used to detect estrogenic and anti-estrogenic substances in sample extracts. This is a recombinant MCF-7 cell line was developed for detection of (anti)estrogenic activities (Demirpence et al, 1993). Since its development, this assay has quickly emerged as a popular technique and had been used by many studies to detect estrogenic activities in wastewater (Snyder et al, 2001; Chen et al, 2004; Yoshimoto et al, 2004). E2 is generally used as a standard for this assay. When testing anti-estrogenic effects, the ER antagonist hydroxytamoxifen (HT) combined with E2 is used as a positive anti-androgen control.

1.3.2 Analytical Chemistry

1.3.2.1 Orbitrap

A recent study successfully detected over 400 pollutants in wastewater using liquid chromatography high-resolution mass spectrometry (Robles-Molina et al, 2014). Similarly, my study aimed to identify and quantify chemical pollutants in our complex samples and closely observe their fate as they move down Swift Current Creek. However, it is often challenging to analyze extremely complex mixtures that contain analytes with a wide variety of chemical and physical characteristics and a wide range of concentrations. This highly difficult task requires an

instrument to have very good performance characteristics including high resolution, high mass accuracy, wide dynamic range, and the capability to perform tandem mass spectrometry.

The Orbitrap is a new type of mass spectrometer invented by Makarov (Hu et al, 2005). The Orbitrap can achieve higher mass resolution and mass accuracy than other laboratory scale analytical mass spectrometers. For example, it can differentiate masses different by only $m/z = 0.0109$, which requires a minimum resolving power of 48,300. In addition, it has a wide dynamic range, with an upper mass limit of $m/z = 5903$. Generally, high mass accuracies of < 10 ppm can be used to identify the chemical formula of detected compounds; the Orbitrap has a mass accuracy of 2.1 ppm (Hu et al, 2005). Based on these enhanced performance features, it was decided to use a Q-Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer from Thermo Scientific to analyze our samples.

1.4 Study Objectives and Hypotheses

The overall goal of this study was to monitor the movement of chemicals as well as assess the biological activities that are released from the Swift Current wastewater treatment plant, including chemicals of toxicological concerns, into Swift Current Creek. Sampling was carried out every month started in June 2011. This study used bioassay directed analysis to assess different chemical activities that may lead to potential environmental effects, followed by using Orbitrap chemical scan to evaluate the chemical composition of Swift Current Creek. Water samples were collected from a total of nine sites along Swift Current Creek including: a railroad bridge upstream of the city of Swift Current and the WWTP (Site 1), WWTP Influent, WWTP Effluent, WWTP Lagoon, Site 5 (4.2 km downstream of the WWTP), Site 8 (21 km downstream of the WWTP), Site 10 (51 km downstream of the WWTP), Site 11 (73 km downstream of the

WWTP), and Site 12 (93 km downstream of the WWTP) (Figure 1-2). The study aimed to assess chemical activities using cell bioassays and construct a chemical profile for each site using Orbitrap instrumental analysis in different seasons to observe seasonal changes in chemical input and potential environmental effects. Thus, the objective of this project was to answer the following questions (null hypothesis listed in parenthesis):

1. Is Swift Current Wastewater treatment plant removing chemicals contaminants at an effective and efficient rate? (H_0 : There are no significant difference in chemical activities between influent and effluent samples).
2. How does the new treatment method compare to lagoon treatment? (H_0 : There are no significant differences in removal efficiencies between lagoon and effluent samples).
3. Are there greater than background levels of potent chemicals present in the creek downstream of WWTP? Are these chemicals input into Lake Diefenbaker through Swift Current Creek? (H_0 : There are no significant difference in chemical concentrations between upstream and downstream sites). If so, which chemicals are input into the lake?
4. Does season have effects on chemical inputs into Swift Current Creek from the WWTP? If so, does season affect total chemical composition in samples (i.e. types of chemicals present in same site among different dates) or does it affect chemical concentrations? (H_0 : There are no significant difference in chemical composition between samples from same site but collected at different times of the year)

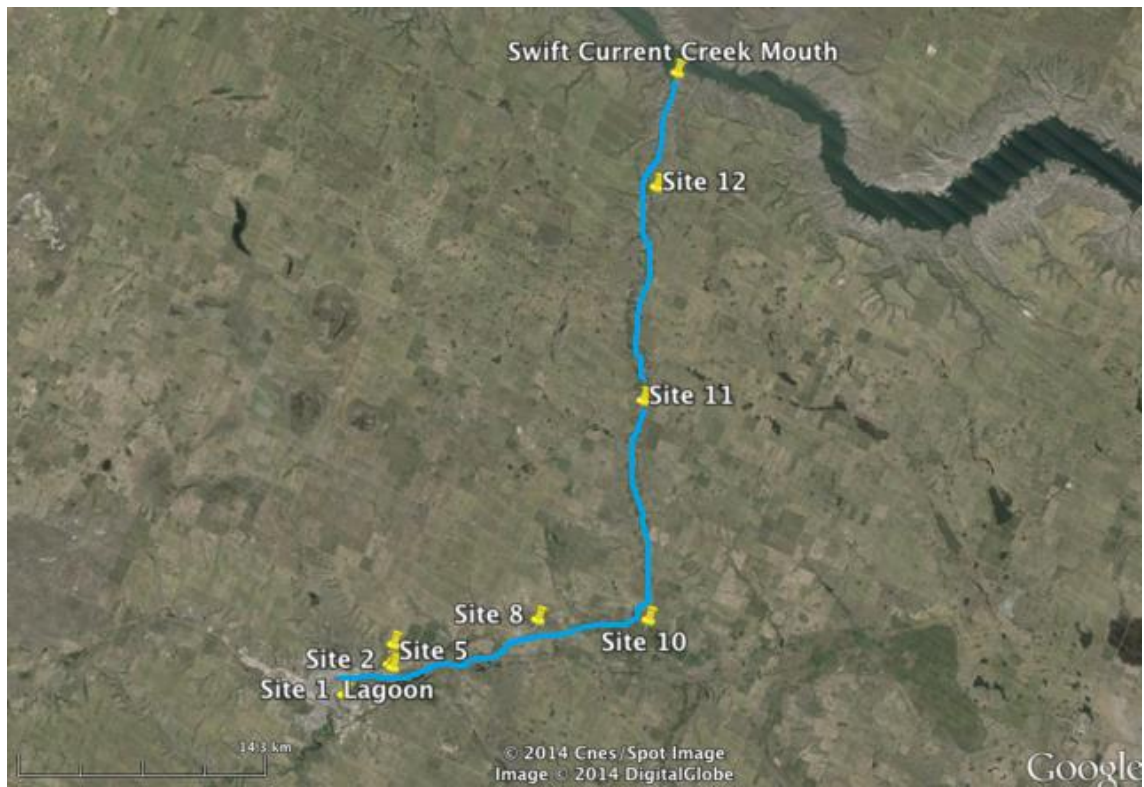


Figure 1-2: Location of Swift Current Creek Sampling Sites

CHAPTER 2: Effect-Directed Analysis (EDA) of Waste and Surface Water Samples from a Northern Prairie River

Preface

This chapter describes the initial steps of the intended effect-directed analysis. This study used different cell assays for pre-screening to detect activities in our samples. By comparing activities in different samples, hypotheses regarding treatment efficiencies as well as temporal and longitudinal changes would be validated. Preliminary instrumental analysis is also presented in this chapter to compliment the cell assay results. This manuscript is intended to be submitted to Environmental Toxicology and Chemistry. The author contributions to this chapter are listed in descending orders:

- Hongda Yuan (University of Saskatchewan) collected, extracted and analyzed contemporary field samples for chapter 2, conducted all statistical analyses and drafted the manuscript. (80%)
- Garry Codling (University of Saskatchewan) assisted in collecting field samples; provided scientific guidance in developing sample extraction methods; provided revisions and comments for the manuscript. (5%)
- Hui Peng (University of Saskatchewan) provided scientific guidance in instrumental analysis of processed field samples; provided revisions and comments for the manuscript. (5%)
- John Giesy, Markus Hecker, and Paul Jones (University of Saskatchewan) provided scientific input and guidance, revised and reviewed this manuscript; procured and provided funding required to conduct this research. (5%)
- Vijay Tumber and Marley Waiser (Environment Canada) assisted in coordinating sample collections, provide historical data and trend for Swift Current Creek. (5%)

2.1 Abstract

A wastewater treatment plant (WWTP) is situated on Swift Current Creek, SK. Although the plant carries out complete secondary treatment followed by UV disinfection, a suite of chemicals is still released into the receiving water. In addition, the City of Swift Current used lagoon treatment for 27 years prior to the construction of the WWTP and chemicals in the lagoon discharges could also pose a potential threat to nearby ecosystems. Swift Current Creek flows into the South Saskatchewan River, which is a major drinking water source for Saskatchewan residents. Thus, we wish to construct a contaminant chemical profile throughout the year to monitor the release and fate of pollutants. Chemical contaminants of interest include hormones, insecticides/pesticides, industrial chemicals and pharmaceuticals and personal care products. A total of 9 sites upstream, downstream and on-site of the WWTP, including from the lagoon, were sampled once or twice a month in 2011 and 2012. Organic compounds were extracted for the purpose of effects-directed analysis. An HPLC Quadrupole-Orbitrap Mass Spectrometer was able to detect and semi-quantify our target chemicals by either full scan or by selected ion monitoring. No PAH activities were detected using H4IIE-*luc* bioassay. Significant reduction (near 100%) of androgenic activities was observed in wastewater effluents and receiving surface water compared to wastewater influents. No estrogenic activities were detected in any samples. However, upon closer observation using the Orbitrap analysis, more longitudinal and seasonal variations in Estradiol Equivalence (EEQ) were observed.

2.2 Introduction

Chemicals released into surface waters are a considerable concern. Chemicals from external sources have the potential to disrupt natural habitat, alter aquatic organisms'

physiological processes, and can potentially lead to human exposure and adverse effects. Endocrine disruptors are major chemicals of concern for the past 40 years, especially when investigating the effects of wastewater in nearby aquatic ecosystems. Endocrine disruptors are foreign chemicals introduced into an organism and affect the endocrine system in four ways: 1) mimic the effects of endogenous hormones; 2) antagonize the effects of endogenous hormone; 3) disrupt the synthesis of endogenous hormones; and 4) disrupt the synthesis of hormone receptors (Sonnenschein and Soto, 1998). Documented endocrine disruptors included some pesticides, natural and synthetic hormones, plasticizers, detergents, surfactants and more (Sonnenschein and Soto, 1998). Although refuted by many, estrogenic compounds have been linked to adverse effects observed on fish and other aquatic species (Purdom et al, 1994; Jobling et al, 1998; Allen et al, 1999). Estrogen agonists are suspected to cause demasculinization in male fish evidenced by increased vitellogenin production, and may affect fish growth, development and reproduction (Johnson and Sumpter, 2001; Segner et al, 2003). In addition to natural and synthetic hormones, many other synthetic chemicals, categorized as Pharmaceuticals and Personal Care Products (PPCPs), also have endocrine disrupting potential (Daughton and Ternes, 1999). PPCPs include a diverse collection of thousands of chemicals including prescription drugs, over-the-counter drugs, cosmetics, fragrances, and other household chemical products. Due to their variety of intended purposes, a wide range of chemicals with different properties are released to the environment. As a result, adverse effects in addition to endocrine disruption may be present, including MXR inhibition, induced antibiotic resistance in pathogens, chronic toxicities in aquatic organisms and general bioaccumulation in tissues (Smital et al, 2004; Berglund et al 2014; Quinn et al, 2008; Du et al, 2015). Furthermore, the adverse effects of a great proportion of known PPCPs still remain to be elucidated.

Major sources of exotic chemicals released into surface water are domestic and agricultural sewage. Pharmaceuticals and endocrine disrupting chemicals are mainly released into surface water from municipal wastewater effluents as they get washed down the drain. Many studies have shown that the removal efficiencies of these chemicals are never 100% during wastewater treatment (Johnson and Sumpter, 2001; Clara et al, 2005; Nakada et al, 2007). On the other hand, agricultural and urban runoffs, concentrated animal feeding operations and landfill leachates also contribute to release of these chemicals as well as pesticides and herbicides to the environment (Benotti et al, 2009). More importantly, contaminated surface waters are serving as a drinking water source that could lead to potential human health concerns. Studies have detected chemical contaminants in drinking water reservoirs as well as treated drinking water (Donald et al, 2007; Benotti et al, 2009). Therefore, the occurrence and potential adverse effects of trace chemicals needs to be closely examined in wastewater effluents and receiving water bodies.

Samples extracted from complex matrices such as wastewater influents and effluents are generally highly complex mixtures containing a vast number of chemicals. Analyses of these samples are usually hindered by the methodology to analyze mixture, the impractical cost for the required standards, and the lack of knowledge of majority of the chemicals (Hecker and Hollert, 2009). Concentrations of each chemical present could range over several orders of magnitude, which makes the development of methodologies accommodating for all compounds nearly impossible (Hecker and Giesy, 2011). To accurately analyze each compound, respective standard should also be analyzed, which could quickly make the study very expensive due to the large number of chemicals to be analyzed. Finally, the lack of information on majority of these chemicals makes the sole concentrations of many chemicals irrelevant in terms of environmental impact (Hecker and Giesy, 2011).

To overcome these challenges, effect-directed analyses (EDAs) were utilized in this study to supplement the chemical analyses. EDAs are generally bioanalytical methods that utilize certain properties of particular chemical groups to elicit biological changes, typically *in vitro* or *in vivo* responses (Hecker and Giesy, 2011). This pre-screening process helps select samples that potentially contains both target or non-target compounds. The next step is fractionation of each sample based on different physical and chemical properties followed by further bioassay. The process is repeated until only a few fractions that elicited biological responses are selected. The goal is to narrow the complex matrix down to a handful of compounds that can be chemically analyzed.

One of advantages of EDA is that the methods are quick and cost-effective in the pre-screening stage, require only 1 or 2 standards that represent the typical agonist and antagonist effects of the biological system. In addition, the elicited biological effect is directly proportional to the overall effect of the mixture, accounting for the concentration and toxic potency of each chemical present without isolating any causal agent (Hecker and Giesy, 2011). Lastly, EDA helps account for effects of unidentified chemicals in a mixture, which as a result provides us a more reliable prediction of the environmental impact. Limitations exist for EDAs as well. Firstly, EDA accounts for effects of unidentified chemicals, but cannot help to pinpoint causal agents of the biological effects (Sanderson et al, 1996). Secondly, many chemical groups lack established EDA methods to assess their biological effects (Hecker and Hollert, 2009). Lastly, sensitivities of EDA methods are not as high as instrumental analytical methods, and highly concentrated samples could be toxic to the biological system (Hecker and Hollert, 2009).

Canada's northern prairie water courses present a unique set of challenges with regard to their use as receiving waters for chemicals. Flows in these water courses are highly seasonal

with spring peak flows; some smaller water courses are seasonally ephemeral. In the winter these waterways are covered in ice and snow thus limiting the availability of light and oxygen. Lastly, seasonal agricultural activities result in high pesticides use that could run off into nearby surface waters. In this study we determine the fate of a variety of contaminant chemicals in a northern prairie water course based on EDA and high resolution mass spectrometry.

2.3 Materials and Methods

2.3.1 Swift Current Wastewater Treatment Plant

The original lagoons treating wastewater for Swift Current SK were constructed in 1955. Discharges from these lagoons were made to Swift Current Creek annually during the spring and fall. Due to population increase, the city developed and opened a new wastewater treatment plant in 2006. The wastewater treatment plant (WWTP) treats 5 million litres of sewage daily for a population of approximately 15,000 people (as of 2011). The plant operates by using primary treatment to filter larger solids followed by secondary activated sludge treatment to breakdown organic matter. The remaining solids are removed via sedimentation and the treated effluent goes through UV disinfection before being released into Swift Current Creek.

2.3.2 Sampling Location and Periods

Sampling was carried out once every month starting in June 2011. Water samples were collected from a total of nine sites along Swift Current Creek including Railroad Bridge upstream of City of Swift Current and WWTP (Site 1), WWTP Influent, WWTP Effluent, WWTP Lagoon, 4.2 km downstream of WWTP (Site 5), 21 km downstream of WWTP (Site 8), 51 km downstream of WWTP (Site 10), 73 km downstream of WWTP (Site 11), and 93 km

downstream of WWTP (Site 12) (Figure 1-2). Water samples were collected into clean, solvent-rinsed 4-L amber solvent bottles and preserved immediately with chloroform. During winter, ice augers were used to drill through ice when weather and conditions permitted.

2.3.3 Sample Preparation

A pre-filter step was applied to eliminate particulates from the water samples. This step facilitates the subsequent extraction processes by preventing the particulates from clogging the extraction cartridges and allowing maximum volume extracted per cartridge. Water samples were filtered by vacuum suction filtration through Macherey-Nagel GF-3 (0.6 μm) filters recommended for wastewater analysis.

Organic chemicals were extracted via solid-phase extraction (SPE) using Waters Oasis® HLB (Hydrophilic-Lipophilic Balance) 6cc 500 mg cartridges. A 2 litre portion of the total 4 litre collected sample was extracted. The method blank was a 2 litre Nanopure water sample collected from the laboratory and extracted for each sampling and extraction period. After extraction, the cartridges were first eluted with 5 mL of methanol followed by 5 mL 1:1 hexane:dichloromethane (DCM) into 15 mL Falcon™ tubes. The solvents of choice covered a wide variety of polarity for the pre-screening purpose. Samples were blown to near dryness under a stream of nitrogen and reconstituted in 250 μL of isooctane. The final concentration of each sample was thus 8,000X more concentrated than the collected sample. Extracted samples were stored at -20 °C in crimp-top vials for chemical analysis. Prior to chemical analysis, samples were diluted to 100X concentration in acetonitrile (ACN) and 10 ng/mL of internal standards were added for recovery analysis for each sample injection.

2.3.4 Effect-Directed Analysis

The EDA approach in this study was to utilize a variety different cell bioassays to screen for biological responses induced by each sample. This study used H4IIE, MVLN, and MDA cell bioassays to assess samples for targeted effects. H4IIE cell assay detects planar hydrocarbons such as polycyclic aromatic hydrocarbons (PAHs), dioxins/furans, and pesticides (Aarts et al, 1995). MVLN and MDA cell bioassays measure estrogenic and androgenic activities respectively (Wilson et al, 2004; Wilson et al, 2002).

2.3.4.1 H4IIE-luc Assay

The H4IIE-*luc* cell line was used to detect PAH and dioxin-like chemicals in the sample extracts. Ninety-six-well culture plates were seeded with 100 μ L of pre-diluted cell suspension to achieve 15,000 cells per well. Cell suspension was not added to the outer perimeter wells due to variable growth in these wells; only PBS was added to these wells to maintain ambient moisture and they were also be used as background luminescence blanks. Dosing solutions were prepared by pre-diluting either 2,3,7,8-TCDD standards or samples in growth media. Final dosing concentrations of TCDD standards were 150 pM, 50.0 pM, 16.7 pM, 5.55 pM, 1.85 pM and 0.617 pM with 0.1% isooctane vehicle. Samples were dosed at 20X concentration with 0.25% isooctane vehicle. Each standard or sample was dosed in triplicates (n=3). Plates were incubated for 24 hours. After exposure period, cells were washed briefly with 75 μ L Dulbecco's Phosphate-Buffered Saline (DPBS with calcium and magnesium). Another 75 μ L of DPBS with Ca^{2+} , Mg^{2+} needed to be added to each well before adding 75 μ L of steadylite plusTM luciferase solution from PerkinElmer. Luciferase activities were measured by luminescence plate reader at room temperature after 15 min incubation in dark and again at 30 min incubation.

2.3.4.2 MDA-kb2 Assay

This study used the MDA-kb2 cell line to detect androgen agonist and antagonist chemicals present in sample extracts. The cells express responses to androgen receptor (AR) agonist Dihydrotestosterone (DHT) at very low concentrations (LOEC = 0.1 nM) (Wilson et al, 2002). In this study, the sensitivity of this assay allowed us to detect as low as 5 pM (0.5 – 1.5 ng/L) of androgenic compounds in surface water. In previous studies, MDA bioassays had demonstrated a wide range of uses in the detection of androgenic and anti-androgenic effects in cosmetic products (Ma et al, 2003) as well as in the effects polybrominated diphenyl ethers (PBDE), a widely used commercial product (Stoker et al, 2005).

Ninety-six-well culture plates will be seeded with 100 µL of pre-diluted cell suspension to achieve 20,000 cells per well. Dosing solutions were prepared by pre-diluting either DHT standards or samples in growth media. The dosing solutions would be used to replace old media in microplates from the Day 1. Final dosing concentrations of DHT standards were 1 nM, 300 pM, 100 pM, 50 pM, 30 pM, 10 pM, and 3 pM with 0.1% isooctane vehicle. Samples were dosed at 20X concentration with 0.25% isooctane vehicle. Each standard or sample was dosed in quadruplicates (n=4). After exposure for 24 hours, cells were washed briefly with 75 µL Dulbecco's Phosphate-Buffered Saline (DPBS with calcium and magnesium). Another 75 µL of DPBS with Ca^{2+} , Mg^{2+} was added to each well before adding 75 µL of steadylite plus™ luciferase solution from PerkinElmer. Luciferase activities was measured by luminescence plate reader at room temperature after 15 min incubation in dark and again at 30 min incubation.

2.3.4.3 MVLN-luc Assay

MVLN assay was first developed by Pons et al in 1990 to characterize estrogenic and anti-estrogenic compounds. Estradiol (E2) is usually used as the standard for this assay, which allows us to assess estradiol equivalency (EEQ) in each sample. MVLN assay had previous been used to detection estrogenic activity in a variety of complex sample matrices including wastewater, sludge and sediment (Snyder et al, 2001; Khim et al, 1999; Koistinen et al, 1998) and it would be very useful in this study as the pre-screening step.

Ninety-six-well culture plates were seeded with 100 μ L of pre-diluted cell suspension to achieve 20,000 cells per well. After 24 hrs of seeding, dosing solutions were prepared by pre-diluting either E2 standards or samples in growth media. Final dosing concentrations of E2 standards were 3 nM, 1 nM, 333 pM, 111 pM, 37 pM, 12 pM, 4 pM and 1.3 pM with 0.1% ethanol vehicle. Samples were dosed at 10X concentration with 0.25% isooctane vehicle. Each standard or sample was dosed in quadruplicate (n=4). After 48 hrs incubation, plates were washed briefly with 75 μ L Dulbecco's Phosphate-Buffered Saline (DPBS with calcium and magnesium). Another 75 μ L of DPBS with Ca^{2+} , Mg^{2+} needed to be added to each well before adding 75 μ L of steadylite plusTM luciferase solution from PerkinElmer. Luciferase activities were measured on a luminescence plate reader at room temperature after 15 min incubation in the dark and again after 30 min incubation.

2.3.4.4 Statistical Analysis

Each replicate represents individual wells receiving the same treatment. Raw Luminescence data were exported as in a spreadsheet. Further modification of the data, including background adjustment and fold change with respect to vehicle control, were performed on

Microsoft Excel. Modified data were then analyzed by one-way ANOVA using IBM SPSS Statistics 23 to examine differences between each site as well between different seasons. Data were analyzed in a generalized linear mixed (GLM) model that included replicates as well as either the collection sites or the collection dates. Statistically significant differences ($p < 0.05$) between each site or difference seasons were calculated using Fisher's least significant difference test during post-hoc analysis.

2.3.5 Orbitrap Chemical Analysis

Extracts were analyzed using a Q Exactive™ mass spectrometer (Thermo Fisher Scientific, Toronto, ON) interfaced to a Dionex™ UltiMate™ 3000 ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific, Toronto, ON). Separation of chemicals was achieved with a Betasil C18 column (5 μ m; 2.1 mm \times 100 mm; Thermo Fisher Scientific, Toronto, ON) with an injection volume of 5 μ l. Ultrapure water (A) and methanol (B) were used as mobile phases. Initially 10% B was increased to 50% in 5 min, then increased to 100% at 20 min and held static for 6 min, followed by a decrease to initial conditions of 10% B and held for 3 min to allow for column re-equilibration. The flow rate was 0.20 mL/min. The column and sample chamber temperatures were maintained at 40 °C and 10 °C, respectively. SIM scans ($m/z = 227.1072, 271.1698, 269.1542, 295.1698$) were recorded at a resolution $R = 35000$ (at m/z 200) with a maximum of 5×10^4 ions collected within 80 ms, based on the predictive automated gain control, with the precursor isolation width set at 2.0 m/z . The general mass spectrometry settings applied for negative ion mode were as follows: spray voltage, 2.7 kV; capillary temperature, 375 °C; sheath gas, 46 L/h; auxiliary gas, 11 L/h; probe heater temperature, 375 °C. Similarly, the settings applied for positive ion mode were: spray voltage, 3.0 kV;

capillary temperature, 400 °C; sheath gas, 46 L/h; auxiliary gas, 15 L/h; probe heater temperature, 350 °C.

2.4 Results and Discussion

2.4.1 Dioxin-Like Activities

Representative sample sites (Upstream, influent, effluent, lagoon, closest downstream, and farthest downstream) from selected sample dates were pre-screened with H4IIE cell bioassay to test for dioxin-like activities. Descriptive statistical values are listed in Table 2-1 and the mean response for all sites was approximately 1-fold. Thus no significant dioxin-like activity was found in Swift Current Creek (Figure 2-1). The preliminary results agreed with the speculations that dioxins and planar hydrocarbons are not the typical chemical contaminants found in municipal wastewater. Furthermore, previous studies utilizing H4IIE-*luc* assay have shown similar low concentrations of PAHs and dioxin-like chemicals in other municipal WWTPs (Ma et al, 2005; Maier et al, 2016). Therefore, no further H4IIE testing was conducted with the remaining samples. However, other studies using H4IIE assay have revealed higher levels of PAHs and dioxin/furans present in wastewater contaminated soils and sediments (Koh et al, 2004; Kannan et al, 2008). Due to the high tendency to partition into soil and sediments for these types of chemicals, concentrations of PAHs and dioxins/furans can accumulate in sediments even if they are only present at very low concentrations in water. Therefore, future sampling and analysis may be required to assess sediments in Swift Current Creek.

2.4.2 Androgenic Activities

Androgenic activities found in Swift Current Creek are summarized in Table 2-2. Aside from very high responses from wastewater influent samples, other samples only induced very close to baseline responses (Figure 2-2a). One-way ANOVA was performed to compare androgenic activities in different sites and only influent samples were significantly different from others samples ($p < 0.005$). A review study by Liu et al had previously summarized that the removal efficiencies of androgenic hormones and their mimics from various studies are close to 100%, supporting the findings in this study (Liu et al, 2009). In addition, a comparative study by Bain et al (2014) using cell bioassays has compared two WWTPs using different treatment strategies and their removal efficiencies. The results supported the phenomenon that androgenic activities, along with other endocrine disrupting activities including estrogen receptor and glucocorticoid receptor, were removed with high efficiencies (Bain et al, 2014). Another study using a Rainbow Trout androgen receptor-binding bioassay also presented results supporting near 100% removal of total testosterone equivalence (Leusch et al, 2006). Therefore, we conclude that androgenic activities were reduced down to baseline level after wastewater treatment, and thus non-detectable androgenic activities were released into Swift Current Creek.

Seasonal variations of concentrations of chemicals in wastewater have been characterized before (Sui et al, 2011). Concentrations are generally based on seasonal usage, with antibacterial drugs found higher concentrations in colder seasons, while bug repellents like DEET were detected in higher concentrations in summer. Since influent samples were the only samples eliciting positive responses, we only performed one-way ANOVA comparing activities in influent samples from different dates to identify any temporal trends (Figure 2-2b). Although significant differences existed between samples ($p < 0.005$), no consistent trends could be

observed and correlated to seasons. These findings suggest that consistent high levels of androgenic hormones and mimics were released into raw sewage throughout the year, but they were also consistently and efficiently removed via treatment. These observations were expected from a municipal treatment plant, as human input of hormones should not be particularly influenced by seasonal changes.

Due to near 100% removal of androgenic activity from the influent, no significant androgenic activities were detected in effluents, lagoon and downstream sites. Thus, no trend could be observed in surface water down the distance of the sampling range. The low level of activities in downstream sites from the cell assays also indicated that no other point source discharge of androgen hormones and mimics were present between the WWTP and where the Swift Current Creek enters Lake Diefenbaker. Furthermore, no temporal differences can be concluded except that androgen activities at each site were consistently low throughout the year. A study has shown that receiving waterbody flow rate may affect contaminant concentrations, as increased flow rate dilutes contaminant concentrations (Loraine and Pettigrove, 2006). The absence of fluctuations in androgenic activities in surface water sites suggested that flow rate did not affect the activities. This further supported that androgenicity was nearly completely removed after treatment and was non-detectable in receiving surface water. Therefore, we concluded that the androgenic activities detected were not affected by season, temperature, flow rate and other possible factors, due to the overall low presence of androgenic hormones and their mimics.

2.4.3 Estrogenic Activities

A pilot study with the MVLN cell bioassay using four representative sampling seasons was conducted. The targeted sampling period, August, September, January, and March each represented late summer, autumn, winter, and early spring respectively (Table 2-3). Anticipated high responses in raw sewage samples, based on the findings from the MDA assay discussed in previous section, were not observed. On the contrary, high estrogenic activities detected in raw sewage using cell assay was widely reported (Bellet et al, 2012; Coors et al, 2004; Bain et al, 2014; Ma et al, 2005). Furthermore, these studies have also reported detectable ranges of estrogen equivalents in final effluents. Upon further careful investigation, we have concluded that dosing the cells at a 10X concentration for influent samples caused cytotoxicity (Figure 2-3a). In addition, only lagoon samples were able to elicit significant but slightly elevated responses, while other samples had shown baseline responses (Figure 2-3a). When examining lagoon samples from different dates, only the March lagoon sample were found to have induced above baseline response while the other three sampled dates induced baseline responses (Figure 2-3b). Based solely on the preliminary assay findings, estrogenic hormones input into raw sewage were very similar to androgenic hormones and did not significantly vary throughout the year. Once again this was possibly due to human input of natural and synthetic hormones into municipal sewage does not dependent on seasonal changes. However, overall lack of activities in these samples hindered us from making any indicative conclusions regarding longitudinal or temporal differences in the creek.

The absence of responses from the MVLN assay had suggested a re-examination of the dose-response standard curve. Using statistical analysis, conclusion was drew that the minimum concentration of estradiol required to induce significant cellular responses was approximately

12.5 ng/L (Figure 2-4). The sensitivity that was determined was much lower than what previous studies have stated (Van den Belt et al, 2004; Gutendorf and Westendorf, 2001). This was possibly due to the residual estradiol present in the dcc-FBS used in the cell assay, which as a result raised the baseline and lowered the sensitivity of the cell bioassay. Unfortunately, this concentration of estradiol could at times be higher than environmental estrogen concentrations (Ternes et al, 1999; Huang and Sedlak, 2001; Benotti et al, 2009), especially for a WWTP that treats sewage for a city as small as Swift Current. Concentrations of estrogen equivalents detected in raw and treated sewage in aforementioned studies were also lower than the cell assay detection limit in this study, ranging from high pg/L to low ng/L (Bellet et al, 2012; Coors et al, 2004; Bain et al, 2014; Ma et al, 2005). Therefore, although results in this study were mostly negative regarding environmental estrogenic activities, it is inconclusive that the WWTP effluent inputs did not influence estrogenic activities in receiving water bodies. Thus, we decided to analyze our samples using Orbitrap HPLC to quantify hormone concentrations in Swift Current Creek at a much higher sensitivity.

2.4.4 Orbitrap Results

For SIM analysis, estrogenic hormone standards (E1, E2, EE2, and BPA) along with deuterated internal standards (E1, E2, and BPA) were injected at controlled concentrations every six samples. Peak areas of internal standards in samples were compared to peak areas of internal standards alone for recovery calculation. Concentrations of estrogenic hormones were calculated from the peak area ratio of each chemical in sample and in standards, while making reference to recovery of each individual sample.

The complex nature of the WWTP influents once again presented challenges for analysis. We experienced poor detection of compounds in WWTP influent samples even with SIM mode which in theory should provide better resolution and reduced noise. To summarize the overall presence of estrogenic activities in the receiving water and be able to reference to our MVLN results, Estradiol Equivalences (EEQ) were used. EEQ was calculated by adding concentrations of E2, EE2, and E1, each multiplied by their corresponding Estradiol Equivalence Factor (EEF) summarized by Jarosova et al (Jarosova et al, 2014). The calculation is described in the equation below:

$$EEQ = [E2] + [EE2] \times EEF_{EE2} + [E1] \times EEF_{E1}$$

The EEQs of our samples analyzed by the Orbitrap are summarized in Table 2-4. Estrogens were non-detectable in influent samples by the Orbitrap, which coincided with no activities shown in the MVLN assay. The results further reiterated the challenges faced when analyzing highly complex mixtures, as the absence estrogenic hormones was more likely due to high noise, not actual absence of estrogenic contaminants. The high background noise in instrumental analysis indicates the complex chemical makeup of the influents, which may have caused cytotoxicity. Another factor leading to poor responses in the bioassay could be interactions of other chemicals that were not detected by instrumental analysis that negated estrogenic activities. In order to isolate active agents, sample clean-up and fractionation was necessary. However, for the scope of our study, we did not proceed with further sample treatment. Nonetheless, we have obtained promising data to support future application of implementing EDA to analyze wastewater and affected surface waters.

The Orbitrap SIM had much more success in analyzing less complex samples, including WWTP effluents. As shown in Figure 2-5, the majority of the samples had an EEQ value less

than 1.25 ng/L, thus they would not induce responses in our MVLN bioassay even if they were dosed at a 10X concentration. The low ng/L concentration range that was detected in effluent samples also agreed with previous studies (Bellet et al, 2012; Coors et al, 2004; Bain et al, 2014; Ma et al, 2005). Although direct comparison of EEQs could not be performed due to absence of data from influent samples, high removal efficiency could still be speculated, based on high complexity of influent samples and low concentrations in effluent samples. The high removal efficiency of current wastewater treatment method was also in accordance to previous literatures (Bain et al, 2014; Liu et al, 2009) In addition, no significant difference was observed between the new secondary treatment method and the conventional lagoon treatment method. Similarly high removal efficiency from both treatment methods was observed in previous studies as well (Servos et al, 2005). However, detectable ng/L ranges of estrogenic chemicals indicated that residual estrogenic hormones were still released into receiving surface water.

Supplementing our MVLN data with the Orbitrap results, it could be observed that the load of estrogenic compounds was diluted as the water flows down the creek. Seasonal pattern was also present, as winter and spring had higher EEQs than summer and fall. The first factor contributing to this phenomenon is that river flow is generally low in winter and high in summer (Loraine et al, 2006). In the case of Swift Current Creek, the surface of the creek froze in winter, thus drastically reducing its flow. Secondly, studies have shown that snow and ice are able to scavenge organic compounds (Daly and Wania, 2004; Wania et al, 1998). When samples were collected in January, digging through the ice layer using an auger could release organic compounds back into the water underneath the ice and potentially be detected in our sample. During March, snowmelts began and more sequestered organic compounds were released back into the water. Lastly, two studies in China also observed similar trends and it could also be due

to lower efficiencies of WWTP's in winter, thus resulting in higher concentrations in early spring (Sui et al, 2011; Yu et al, 2013). All of these phenomenon could have caused the observed spiking in chemical concentration as well as in estrogenic activity.

2.5 Conclusions

Cell bioassays were proven to be a cost-effective and quick means to pre-screen and analyze complex sample matrices. In an effect-directed analysis testing for interactive toxic effects, these cell bioassays were the first and most indicative step to isolate samples illustrating target effects. Batteries of cell assays represent widely accepted methods to screen for different types of chemical contaminants (Bellet et al, 2012; Coors et al, 2004; Bain et al, 2014; Ma et al, 2005). The preliminary study with H4IIE cells quickly eliminated planar hydrocarbons such as PAHs and dioxins as potential contaminants from the WWTP. However, these chemicals may be present in sediments in the Swift Current Creek watershed and may require further analysis. MDA cell assays revealed near 100% removal of androgenic activities from wastewater influents. Due to low presence of androgenic hormones in effluents and consequently in receiving surface water, no androgenic activities could be detected in samples collected downstream of the WWTP. The results agreed with previous studies determining removal efficiencies of androgenic hormones and mimics from wastewater, thus eliminating androgen agonists from chemicals of concern. The MDA cell assay is also able to provide an alternative approach when direct instrumental analysis for androgenic compounds becomes a challenge.

It was observed in this study that sensitivity dictates the application of certain cell bioassays for environmental assessment, as the limit of detection needs to be close to relevant environmental concentrations. The MVLN assay has been long used to assess estrogenic effects

and has a higher sensitivity compare to other assays (Gutendorf and Westendorf, 2011). However, due to high background level of estradiol, the results from the assay alone was inconclusive. A newly developed T47D cell bioassay is able to detect estradiol equivalence of as low as 1 ng/L in effluent samples. Such high sensitivity is helpful in assessing environmental impact, especially for chronic adverse effects potentially caused by the low but consistent presence of chemical contaminants. A European project known as “Identification of Endocrine Disrupting Effects in Aquatic Organisms (IDEA)” had determined that EE2 was able to alter fertilization success and vitellogenin induction in zebrafish at a concentration as low as 1.67 ng/L (Segner et al, 2003). Furthermore, the same study showed that at an EE2 concentration of 10 ng/L, reproduction was completely inhibited in zebrafish. While the T47D bioassay would be capable to reveal concerning activities at this level, the MVLN bioassay in contrast would not be able to pick up the signal. However, as determined in this study, a bioassay’s sensitivity not only relies on the cell line, but is also dictated by the incubating environment. Many factors could still greatly reduce the sensitivity of an assay and render the results irrelevant. Nonetheless, the effect-directed analysis of coupling cell bioassay with instrumental analysis is a very promising method to monitor treated and untreated sewage, where hundreds of chemicals with unknown toxicity are present.

2.6 Tables and Figures

Table 2-1: Dioxin-like activities detected in Swift Current Creek using H4IIE cell bioassay. Mean fold change value was calculated from replicates of all three sampling dates (July 2011, August 2011 and January 2012). Final concentration of samples in each incubation well is 20X concentrated.

Sampling Sites	N	Mean Fold Change	Std. Deviation
Site 1	9	1.06	0.12
Influent	9	0.91	0.11
Effluent	9	0.97	0.23
Lagoon	9	0.98	0.11
Site 5	9	1.02	0.13
Site 12	9	1.11	0.20

Table 2-2: Androgenic activities detected in Swift Current Creek using MDA cell bioassay. Mean fold change value was calculated from replicates of all tested sampling sites. Final concentration of samples in each incubation well is 20X concentrated.

Sampling Sites	N	Mean Fold Change	Std. Deviation
Site 1	48	1.22	0.30
Influent	32	4.94	0.91
Effluent	40	1.23	0.26
Lagoon	40	1.14	0.34
Site 5	44	1.11	0.32
Site 8	24	1.41	0.33
Site 10	24	1.38	0.32
Site 11	28	1.36	0.28
Site 12	40	0.97	0.33

Table 2-3: Estrogenic activities detected in Swift Current Creek using MVLN cell bioassay. Mean fold change value is calculated from replicates of all tested sampling sites. Final concentration of samples in each incubation well is 10X concentrated.

Sampling Sites	N	Mean Fold Change	Std. Deviation
Site 1	16	0.97	0.16
Influent	16	0.69	0.35
Effluent	16	1.01	0.11
Lagoon	16	1.17	0.25
Site 5	16	0.95	0.14
Site 8	16	0.94	0.18
Site 10	16	0.93	0.14
Site 11	16	1.00	0.13
Site 12	16	1.02	0.26

Table 2-4: Estradiol equivalence found in Swift Current Creek. Estradiol equivalence is the sum of concentrations of E2, E1 and EE2 analyzed by Orbitrap, adjusted by the estradiol equivalence factor determined by MVLN cell bioassay (E1 = 0.01, EE2 = 1.25, E2 = 1). Influent samples had poor recovery due to highly complex matrix. Although calculated concentrations in influent samples do not reflect true concentrations, the poor recoveries help explain cell toxicity and/or nullifying effects observed in the MVLN bioassay.

Sample Date	Estradiol Equivalence (ng/L)			
	2011-08-01	2011-09-26	2012-01-09	2012-03-05
Site 1	<0.05	<0.05	<0.05	<0.05
Influent	<0.05	<0.05	0.46	0.07
Effluent	0.31	0.10	0.34	2.26
Lagoon	<0.05	<0.05	<0.05	<0.05
Site 5	<0.05	0.37	<0.05	2.26
Site 8	<0.05	0.35	2.23	0.89
Site 10	<0.05	<0.05	1.48	0.66
Site 11	0.87	<0.05	0.89	1.10
Site 12	0.72	<0.05	<0.05	3.68

Dioxin-Like Activity Detected in Swift Current Creek using H4IIE-luc Cell Bioassay

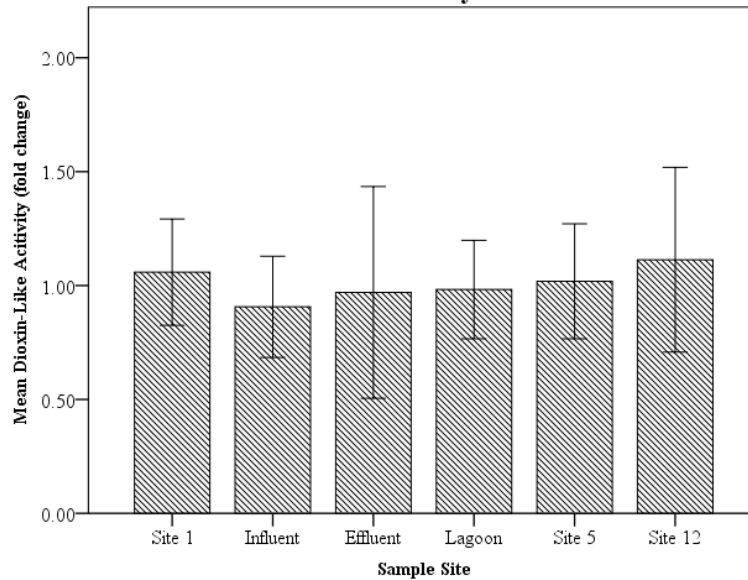


Figure 2-1: Dioxin-like activities detected in Swift Current Creek in July 2011, August 2011, and January 2012. Each column represents the mean fold change in each sampling site from all three sampling dates. No significant difference can be observed from different sampling dates as well as between different sampling sites.

Androgenic Activities detected in Swift Current Creek using MDA-kbluc cell bioassay

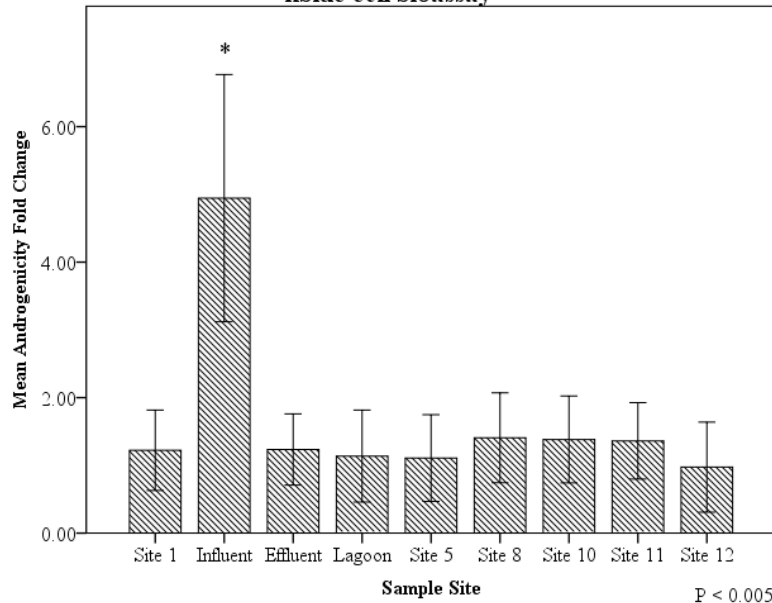


Figure 2-2a: Androgenic activities detected in Swift Current Creek using MDA cell bioassay. Each column represents the mean fold change in each sampling site from all tested sampling dates. Influent samples shown significantly elevated responses (one-way ANOVA, $p < 0.005$), which is expected. Other sampled sites did not elicit any significant responses.

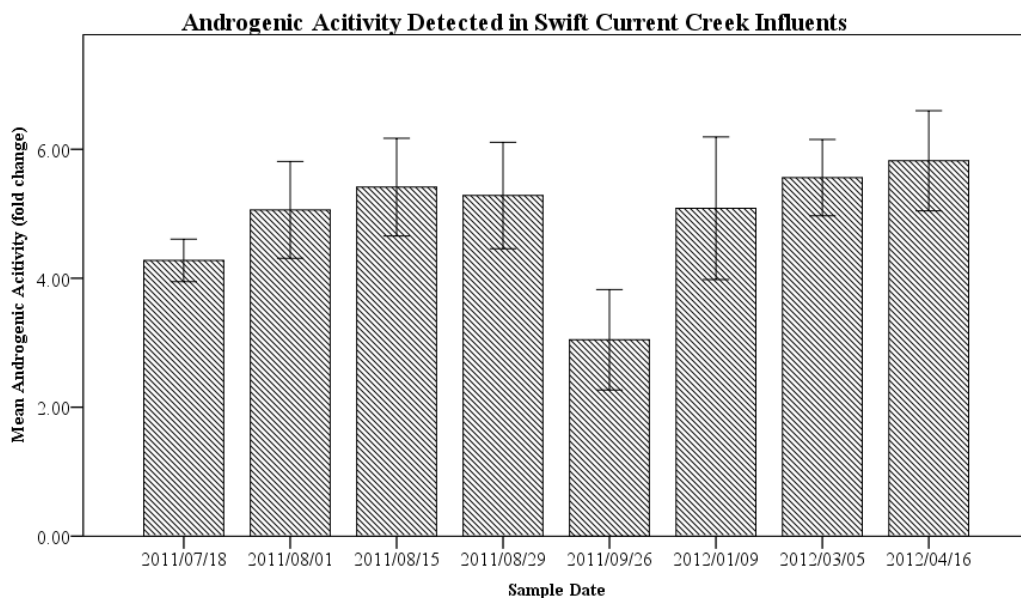


Figure 2-2b: Androgenic activities found in influent samples during different seasons. Seasonal variations are evident, with cellular responses from different sampling dates significantly different from each other (one-way ANOVA, $p < 0.005$).

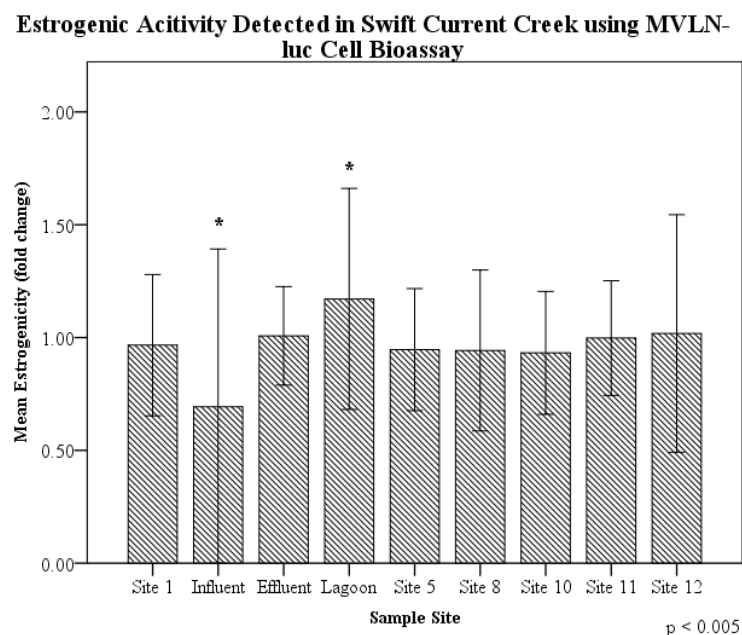


Figure 2-3a: Estrogenic activities detected in Swift Current Creek. Each column represents the mean fold change in each sampling site from all tested sampling dates. Influent samples shown significantly reduced responses (one-way ANOVA, $p < 0.005$), possibly due to cell stress/death due to cytotoxicity. Lagoon samples showed significant, although slightly, elevated responses (one-way ANOVA, $p < 0.005$).

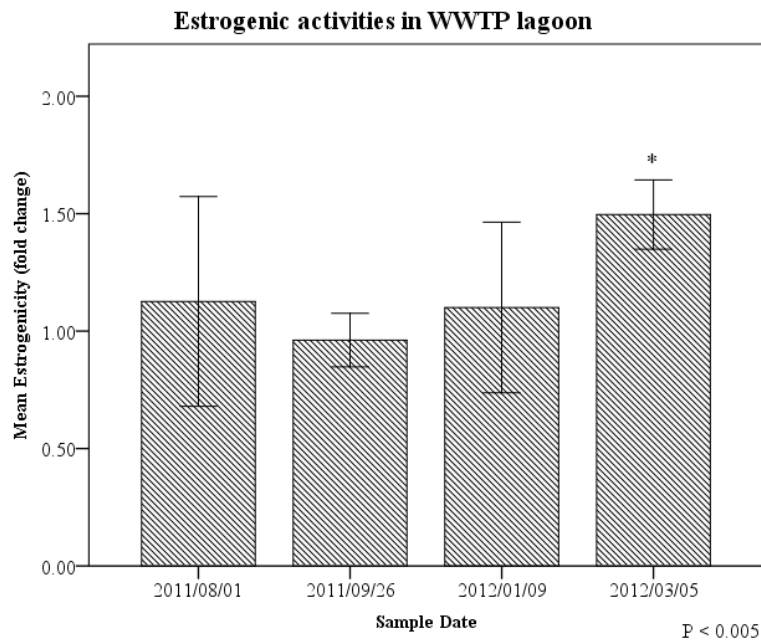


Figure 2-3b: Estrogenic activities found in lagoon samples during different seasons. Lagoon samples had significant estrogenicity detected, with the March sample having significantly higher cellular responses than other sampling dates (one-way ANOVA, $p < 0.005$).

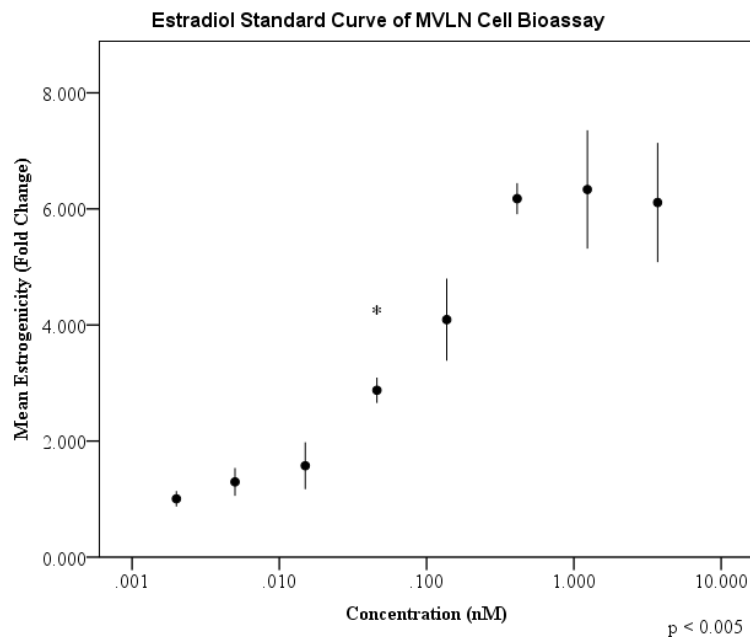


Figure 2-4: Standard curve of MVLN cell bioassay dosed with Estradiol. Significant elicitation above baseline started at approximately 0.046 nM, which is around 12.5 ng/L (one-way ANOVA, $p < 0.005$). High baseline is likely due to high background level of estradiol present in DCC-FBS. Therefore, DCC-FBS has great influence on the sensitivity of MVLN cell bioassays.

Estradiol Equivalency found in Swift Current Creek

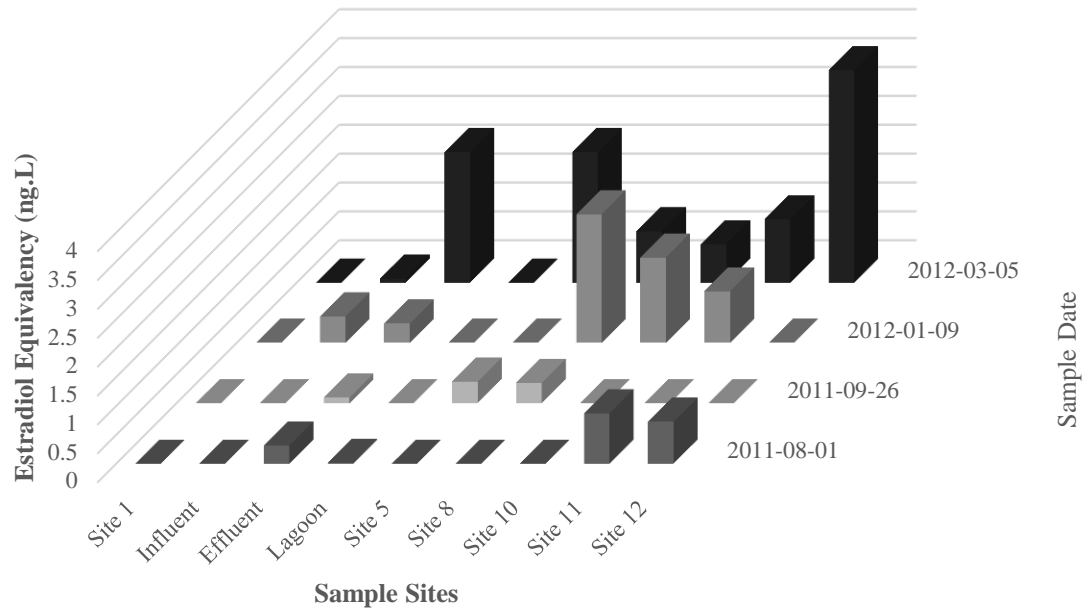


Figure 2-5: Estradiol equivalencies (EEQ) found in Swift Current Creek from the four sampling dates tested with the MVLN cell bioassay. Estradiol equivalency in this study is the sum of concentrations of E2, E1 and EE2 adjusted with Estradiol Equivalency Factors.

CHAPTER 3: The Fate of “Exotic” Chemicals from Wastewater Effluent Released to a Small Northern Prairie River

Preface

Upon examining cell bioassay results, it was concluded that cell assays were sufficient to provide a snapshot of chemical activities found in raw/treated sewage and surface waters. However, latter section of Chapter 2 had raised concerns regarding cell assays’ sensitivities as well as their inability to provide more detailed chemical analysis. Thus Chapter 3 presents results from instrumental analysis using Orbitrap Hi-Res LC-MS and more in-depth examinations of exotic chemicals in Swift Current Creek. The results had revealed variable removal efficiencies for the compounds we have examined and interesting seasonal patterns in downstream waterbody. This manuscript is intended to be submitted to Environmental Toxicology and Chemistry. The author contributions to this chapter are listed in descending order:

- Hongda Yuan (University of Saskatchewan) collected, extracted and analyzed contemporary field samples for chapter 2, conducted all statistical analyses and drafted the manuscript. (75%)
- Garry Codling (University of Saskatchewan) assisted in collecting field samples; provided scientific guidance in developing sample extraction methods; provided revisions and comments for the manuscript. (10%)
- Hui Peng (University of Saskatchewan) provided scientific guidance in instrumental analysis of processed field samples; provided revisions and comments for the manuscript. (5%)
- John Giesy, Markus Hecker, and Paul Jones (University of Saskatchewan) provided scientific input and guidance, revised and reviewed this manuscript; procured and provided funding required to conduct this research. (5%)
- Vijay Tumber and Marley Waiser (Environment Canada) assisted in coordinating sample collections, provide historical data and trend for Swift Current Creek. (5%)

3.1 Abstract

A wastewater treatment plant (WWTP) is situated on Swift Current Creek, SK. Although the plant carries out complete secondary treatment followed by UV disinfection, a suite of industrial and PPCP chemicals is still released into the receiving water. In addition, the City of Swift Current used lagoon treatment of sewage for 27 years prior to the construction of the WWTP and the lagoons may also represent a source of chemicals to nearby ecosystems. Swift Current Creek drains to the South Saskatchewan River system, which is a major drinking water source for Saskatchewan residents. Our aim was to construct a temporal and spatial profile of the anthropogenic chemicals in Swift Current Creek to investigate the fate of pollutants. Chemical contaminants of interest included hormones, insecticides/pesticides, industrial chemicals and pharmaceuticals and personal care products. A total of 9 sites including upstream, downstream and on-site of the WWTP and its lagoons, were sampled once or twice a month in 2011 and 2012. Orbitrap ultrahigh resolution mass spectrometry was used to detect and quantify target chemicals by either full scan or by selected ion monitoring. Significant decreases in the concentrations of chemicals were observed in WWTP effluent samples compared to influents. Concentrations from sampling sites downstream of the WWTP were in most cases further decreased. Seasonal variations in chemical concentrations were seen to be caused by water flow, seasonal usage, as well as seasonal occurrence. While highly removed hormones remained in low concentrations throughout the year, some PPCPs were more persistent and exhibited different concentrations during different seasons.

3.2 Introduction

The release of anthropogenic chemicals to receiving surface waters has always been a concern. Pesticides that enter nearby waterbodies through leaching and runoff can affect non-target aquatic organisms. For example, studies had shown that many pesticides can inhibit organisms' MXR mechanisms, the first line of defence against toxic chemicals (Smital et al, 2004). Herbicides usually target an organism's photosystem, and when flushed into nearby surface water, they can also affect algal species (Peterson et al, 2004; Tang and Escher, 2014).

In addition to pesticides, endocrine disruptors are contaminants of emerging concern in aquatic ecosystems. Endocrine disruptors are foreign chemicals introduced into an organism and affect the endocrine system in four ways: 1) mimic the effects of endogenous hormones; 2) antagonize the effects of endogenous hormone; 3) disrupt the synthesis of endogenous hormones; and 4) disrupt the synthesis of hormone receptors (Sonnenschein and Soto, 1998). Documented endocrine disruptors included some pesticides, natural and synthetic hormones, plasticizers, detergents, surfactants and more (Sonnenschein and Soto, 1998). Studies have indicated that estrogenic compounds have adverse effects on fish and other aquatic species (Purdom et al, 1994; Jobling et al, 1998; Allen et al, 1999). Estrogen agonists are suspected to cause demasculinization in male fish resulting in increased vitellogenin production, which has the potential to affect fish reproduction and growth (Johnson and Sumpter, 2001; Segner et al, 2003). In addition to natural hormones, synthetic chemicals categorized as Pharmaceuticals and Personal Care Products (PPCPs) can also have endocrine disrupting potential (Daughton and Ternes, 1999). Due to the wide range of chemical properties of PPCPs, in addition to endocrine disruption, other adverse effects may also include MXR inhibition, induced antibiotic resistance in pathogens, chronic toxicities in aquatic organisms and general bioaccumulation in tissues

(Smital et al, 2004; Berglund et al 2014; Quinn et al, 2008; Du et al, 2015). Furthermore, many of the effects of various PPCPs still remain unknown and/or uninvestigated.

Pharmaceuticals and similar endocrine disrupting chemicals are mainly released into surface water from municipal sewage discharges. Many studies have shown that these chemicals are not completely removed during wastewater treatment (Johnson and Sumpter, 2001; Clara et al, 2005; Nakada et al, 2007). In addition, agricultural and urban runoffs from concentrated animal feeding operations and landfill leachates can also contribute to the release of these chemicals as well as pesticides and herbicides to the environment (Benotti et al, 2009). More importantly, many surface waters also serve as drinking water sources for humans and livestock. Studies had detected chemical contaminants in drinking water reservoirs as well as treated drinking water (Donald et al, 2007; Benotti et al, 2009). Therefore, the occurrence and behaviour of trace chemicals in wastewater effluents and receiving waters needs to be closely examined.

The South Saskatchewan River is 1392 km long and originates in southern Alberta. The catchment is impounded at Lake Diefenbaker, which is located 100 km southwest of Saskatoon and is a valuable freshwater source for both local and downstream communities (North et al, 2014). Approximately 45% of the total Saskatchewan population relies on the South Saskatchewan River for their daily water needs (North et al, 2014). The South Saskatchewan River receives chemical and waste discharges throughout its course, ranging from municipal and industrial point source discharges, feed lots, and non-point source agricultural runoff.

A major point source for chemical releases to Lake Diefenbaker is the Swift Current wastewater treatment plant (WWTP) which was constructed in 2006. The WWTP treats 5 million litres of sewage daily for a population of approximately 15,000 people (as of 2001). The plant operates using primary treatment to filter larger solids followed by secondary activated

sludge treatment to breakdown organic matter. The remaining solids are removed by sedimentation and the effluent undergoes UV disinfection before being released to Swift Current Creek. This study quantifies inputs and fates of a variety of chemical compounds, mainly from the WWTP, entering Swift Current Creek, a tributary of the South Saskatchewan River system.

3.3 Materials and Methods

3.3.1 Sampling Location and Periods

Sampling was carried out once a month starting in June 2011. Water samples were collected from a total of nine sites along Swift Current Creek including Railroad Bridge upstream of the city of Swift Current and the WWTP (Site 1), WWTP Influent, WWTP Effluent, WWTP Lagoon, Site 5 (4.2 km downstream of the WWTP), Site 8 (21 km downstream of the WWTP), Site 10 (51 km downstream of the WWTP), Site 11 (73 km downstream of the WWTP), and Site 12 (93 km downstream of the WWTP, all sites shown in Figure 1-2). Water samples were collected in clean, solvent-rinsed 4 L amber solvent bottles and preserved immediately with chloroform. During winter, an ice auger was used to drill through ice when weather and ice conditions permitted.

3.3.2 Sample Extraction

Samples were pre-filtered to eliminate particulates thereby preventing clogging of the SPE extraction cartridges and allowing maximum volumes to be extracted per cartridge. Water samples were filtered by vacuum filtration through Macherey-Nagel GF-3 (0.6 μm) filters recommended for wastewater.

All organic chemicals were extracted from filtered water samples by solid-phase extraction (SPE) using Waters Oasis® HLB (Hydrophilic-Lipophilic Balance) 6cc 500 mg cartridges. A 2 L portion of the total 4 L sample was extracted. A 2 litre Nanopure water sample collected from the laboratory was also extracted for each sampling period to serve as the method blank control. The cartridges were first eluted with 5 mL of methanol followed by 5 mL 1:1 hexane:dichloromethane (DCM) into 15 mL Falcon™ tubes. Samples were then blown down to near dryness under a stream of nitrogen and reconstituted in 250 µL of isooctane. The final concentration of each sample was therefore 8,000X more concentrated than the collected sample. Extracted samples were stored at -20 °C in crimp-top vials for chemical analysis. Prior to chemical analysis, samples were diluted to 100X concentrated in acetonitrile (ACN) and 10 ng/mL of internal standards were added for recovery analysis for each sample injection.

3.3.3 Chemical Standards

A series of chemicals were purchased as authentic standards (Table 3-1). This list was compiled based on local abundance, potential risk, and availability of authentic standards.

3.3.4 Orbitrap Chemical Analysis

For this study, four sets of sample extracts were analyzed; August, September, January and March were selected to reflect changes to season as well as agricultural activities. Extracts were analyzed using a Q Exactive™ mass spectrometer (Thermo Fisher Scientific, Toronto, ON) interfaced to a Dionex™ UltiMate™ 3000 ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific, Toronto, ON). Separation of chemicals was achieved with a Betasil C18 column (5 µm; 2.1 mm × 100 mm; Thermo Fisher Scientific,

Toronto, ON) with an injection volume of 5 μ L. Ultrapure water (A) and methanol (B) were used as mobile phases. Initially 10% B was increased to 50% in 5 min, then increased to 100% at 20 min and held static for 6 min, followed by a decrease to initial conditions of 10% B and held for 3 min to allow for column re-equilibration. The flow rate was 0.20 mL/min. The column and sample chamber temperatures were maintained at 40 °C and 10 °C, respectively. Data were acquired using full scan mode and selected ion monitoring (SIM). Briefly, MS scans (100 – 1000 m/z) were recorded at resolution $R = 70000$ (at m/z 200) with a maximum of 3×10^6 ions collected within 200 ms, based on the predictive automated gain control. SIM scans ($m/z = 227.1072, 271.1698, 269.1542, 295.1698$) were recorded at a resolution $R = 35000$ (at m/z 200) with a maximum of 5×10^4 ions collected within 80 ms, based on the predictive automated gain control, with the precursor isolation width set at 2.0 m/z . The general mass spectrometry settings applied for negative ion mode were as follows: spray voltage, 2.7 kV; capillary temperature, 375 °C; sheath gas, 46 L/h; auxiliary gas, 11 L/h; probe heater temperature, 375 °C. Similarly, the settings applied for positive ion mode were: spray voltage, 3.0 kV; capillary temperature, 400 °C; sheath gas, 46 L/h; auxiliary gas, 15 L/h; and probe heater temperature, 350 °C.

3.3.5 Spike Recovery Experiments

Extraction efficiencies were determined from a separate spike recovery experiment. Three matrices (influent, effluent and river samples) representative of the collected samples were studied. Samples from each matrix were extracted following the same method as outlined. Non-spiked extracts were first analyzed using the same standards. Pre-existing chemical concentrations in each matrix were calculated to serve as background. Compound mixtures were then spiked in each matrix in triplicates, with concentrations for each standard approximately ten

times greater than pre-existing concentrations or maximum allowed concentrations. Recovery was calculated by comparing the detected concentration of each chemical to the expected concentration.

3.3.6 Statistical Analysis

For SIM analysis, estrogenic hormone standards (E1, E2, EE2, and BPA) along with deuterated internal standards (E1, E2, and BPA) were injected at controlled concentrations every six samples. Peak areas of internal standards in samples were compared to peak areas of internal standards alone for recovery calculation. Concentrations of estrogenic hormones were calculated from the peak area ratio of each chemical in sample and in standards, while making reference to recovery of each individual sample.

For full-scan analysis, a standard mixture containing 31 chemicals was injected at 500, 100, 50, 20, 10, 5, 1, 0.1, and 0.01 ng/mL to construct calibration curves. Internal standards containing D3 naproxen, D3 caffeine and D3 DEET at 10 ng/mL were present in each standard mixture for recovery analysis. The 50 ng/mL standard was also injected after every six samples for concentration calculation. Chemical concentrations in each sample were calculated using the 50 ng/mL standard closest in time as reference.

3.4 Results and Discussion

3.4.1 Spike Recovery

Spike recovery results are summarized in Table 3-2 below. Samples were analyzed using full m/z scan mode only. Certain compounds, mainly hormones including E2 and estrone, had poor detection on the instrument in full scan mode. Influent and river samples demonstrated

large differences in recovery. The vast difference in the make-up and the content of each matrix should be responsible for the observed phenomenon. The treated effluent was expected to be less complex than raw sewage, yet more complex than river water. As a result, some chemicals in effluents demonstrated similar poor recoveries as influents (carbamazepine and diazepam) while others chemicals resembled the more complete recoveries seen for the river water samples (pentachlorophenol and progesterone).

3.4.2 Pesticides found in Swift Current Creek

Two major biocides, atrazine and chlorpyrifos, were detected in water samples (Table 3-3). Concentrations of these two compounds were similarly low in both influents and effluents (ranged from 0.2 – 3.8 ng/L and below detection limit to 1.3 ng/L for atrazine and chlorpyrifos respectively). A possible source of low input of these pesticides into municipal wastewater could be residential or domestic uses as well as potential residues from food (Department of Public Health and Human Services, 2003). Atrazine is also widely used for lawn weed treatment while chlorpyrifos is used to treat pests on pets. Therefore trace amount could also be present in municipal sewage from laundry and other cleaning wastes. Concentrations of these two compounds in surface waters were greater than in effluents, supporting the hypothesis that leaching and runoff of pesticides from agricultural lands may be the major source to nearby surface waters.

Atrazine levels in surface water samples ranged from below the detection limit to 6.5 ng/L. Atrazine is widely detected in environmental samples from the Canadian Prairies, namely Saskatchewan and Manitoba, compared to the neighboring province of Alberta (Donald et al, 2007). Atrazine was detected in the majority of surface water samples, which agrees with

previous findings supporting atrazine being one of the most common environmental contaminants found in surface waters near agricultural land (Benotti et al, 2009; Donald et al, 2007). In the study by Donald et al (2007), 15 communities in Manitoba, Saskatchewan, and Alberta were investigated. Two insecticides, twenty-seven herbicides and two degradation products were detected in 15 reservoirs at total concentrations from 48 ng/L to 1,062 ng/L. However, atrazine concentrations detected in the current study were lower than other studies. The lower concentrations of atrazine detected in our study was possibly due the timing of our sampling, which did not overlap with the farming season (i.e. pesticide application period) and thus atrazine was not used in larger quantities. There was also no longitudinal variation in the creek, as concentration of atrazine in downstream sites were consistently low (Figure 3-1). Consistent concentrations down the creek also eliminated the possibility of additional point source discharge into the creek through leaching or runoff from nearby farmlands.

3.4.3 PPCPs found in Swift Current Creek

Seven of the targeted PPCPs were detected at wide concentration ranges in the water samples (Table 3-4). The observed trends in concentration ranges and removal efficiencies corresponded very well with recent studies yet demonstrated differences among compounds (Sui et al, 2011; Miao et al, 2005; Focazio et al, 2008; Aronson et al, 2012; Benotti et al, 2009;). Caffeine (beverages), DEET (insect repellent) and triclosan (antimicrobial found in detergents), which are commonly found in municipal sewage as daily usage chemicals, exhibited great differences in concentrations between influent and effluent samples. The results demonstrated that not only relatively high amounts of these chemicals were input into raw sewage, they were also highly efficiently removed from sewage through wastewater treatment. Other studies also

supported these findings by demonstrating similar efficiencies of the current wastewater treatment methods at removing of these PPCPs (Sui et al, 2011; Aronson et al, 2012). However, due to high input in raw sewage, the very small proportion that remained in treated sewage still resulted in low but significant concentrations of these chemicals detected in effluents and in surface waters downstream of the WWTP. Although the toxicities of these compounds are still unknown, significant input of these compounds could raise concerns about the potential effects on the downstream aquatic environment.

In contrast with the chemicals discussed above, some other PPCPs were more persistent in municipal sewage. In our study, carbamazepine, clofibrate and trimethoprim exhibited similar concentrations in both influents and effluents. Similarly, poor removal efficiencies of these compounds were also reported in other studies (Sui et al, 2011; Petrovic et al, 2003). In addition, studies on carbamazepine, an antiepileptic drug commonly found in municipal sewage, and its metabolites have concluded that not only is carbamazepine persistent throughout treatment, its metabolites are also detected at relatively great concentrations in both influents and effluents (Miao et al, 2005; Leclercq, 2009). On the other hand, although gemfibrozil illustrated a relatively great removal efficiency (84%), due to its high input, the residual proportion still yielded relatively great concentrations in effluents and lagoon samples.

The most probable explanation for this observation is that some pharmaceuticals are designed to resist biodegradation in order to reach their intended therapeutic site of action. During the treatment process, many WWTPs implement activated sludge methods which rely on microbial biodegradation of organic chemicals. Since biodegradation rates for chemicals are variable, some resistant pharmaceuticals can be released into nearby surface waters and can be detected in significant quantities as shown in this study. Furthermore, improved treatment

technologies did not drastically increase removal efficiency, as comparable removal rates were observed between conventional activated sludge methods and newer membrane bioreactors (Clara et al, 2005). Therefore, it appears that wastewater treatment has reached a plateau with regard to the removal of a variety of persistent PPCPs. The relatively high persistence of certain PPCPs during treatment combined with the consistent rates of input equal to or exceeding rates of removal and degradation lead to pseudo-persistence of PPCPs in aquatic environments. PPCPs definitely pose environmental concerns and their toxic effects need to be further investigated.

3.4.4 Hormones found in Swift Current Creek

Several estrogenic hormones were detected in the water samples (Table 3-5). These hormones had been frequently detected in Canadian sewages in other studies (Ternes et al, 1999; Carballa et al, 2004). Moreover, several studies had detected similar ng/L concentrations ranges of both E2 and EE2 in effluents in different countries (Ternes et al, 1999, Huang and Sedlak, 2001). Last but more importantly, many adverse effects observed and reported in aquatic environments were associated with E2 and EE2 at ng/L ranges. Unfortunately, as seen in this study, due to the complex composition of the wastewater influents and effluents the detection and quantitation of these hormones was problematic, particularly for WWTP influents.

Efficient yet incomplete removal of these hormones post-treatment was observed in the effluents (85% average removal reflected in this study). Moreover, concentrations found in effluents are similar to those found in lagoon samples, further suggesting improved and updated treatment methods do not necessarily improve removal efficiency of many compounds. In this study, no significant difference was observed between the new secondary treatment method and the conventional lagoon treatment method. Comparable high removal efficiency from both

treatment methods was observed in previous studies as well (Servos et al, 2005). Two major factors were hypothesized to be affecting hormone behaviours in effluent and lagoon samples: 1) hormones were allowed more time to bind to particulates and sediments when released into the lagoon or 2) hormones were allowed more time to be degraded in the lagoon compared to secondary treatment (months to years vs. hours to days). The similarity in concentrations detected in the effluent and lagoon samples overturned the proposed theory and suggested that hormones do not bind to particulates nor do they degrade in lagoon over time. Such properties of these hormones raise further concerns about their potential influence in the aquatic environment as they seem to be more bioavailable to aquatic organisms.

It was worthy of note that hormone concentrations detected in WWTP effluents were above background levels compared to upstream surface water. Although detected concentrations appeared diluted once released into the surface water and travelled downstream of the WWTP, potential concerns could still arise during both dry months and the mid-winter months when effluent comprises a significant fraction of the flow in Swift Current Creek. Concentrations of hormones detected in the surface water samples agreed well with previous studies (Benotti et al, 2009; Huang and Sedlak, 2011; Waiser et al 2011). Most downstream sites were non-detect with only a few samples showing detections in ng/L range (Figure 3-2). Site 12 demonstrated consistent higher concentrations of estrogenic hormones compared to other upstream sites. We hypothesize that there was likely point source discharges other than the WWTP releasing hormones down the creek. It was noted that livestock were seen downstream by the creek and it was highly possible that the hormones detected in Site 12 was from the livestock.

3.4.5 Seasonal Variations in Swift Current Creek

Based on our findings and previous research, it was hypothesized that seasonal variations in the creek would be mostly dictated by the following factors: 1) presence and occurrence in effluents, 2) receiving water body flow rates, and 3) seasonal usage and/or occurrence of certain chemicals. The flow of most rivers is generally at its lowest in winter and gradually increases as the weather warms up and peaks in summer (Loraine et al, 2006). The same flow rate trend was assumed for Swift Current Creek. On the northern prairies, herbicides and pesticides are applied mainly in the spring/summer growing season. However, the flow in Swift Current Creek is also much higher in the spring compared to the late summer to winter. As a result, concentrations of pesticides detected in Swift Current Creek were rather consistent during our analyzed sampling period. Note that the analyzed sampling period incidentally avoided the peak of pesticide use, which is early summer. In addition, pesticides were detected at much lower concentrations compared to other studies and thus seasonal variations were not noticeable nor conclusive. Hormones are expected to be found more consistently in wastewater influents and effluents throughout the year. Therefore, during low-flow seasons, higher concentrations are found in the receiving water body (Figure 3-3). During low flow winter periods, livestock would be less active near the creek and thus decreased input of hormones into the creek. As a result, similar to pesticides, low chemical input coincided with low flow rate and resulted in consistent concentration of hormones in Swift Current Creek.

PPCPs demonstrated different seasonal trends due to various removal efficiencies. Caffeine and DEET, due to their high removal efficiencies and low concentrations in effluents and surface water, showed minimal temporal variations in concentrations. Aronson et al had reported that some studies had observed temporal variation of DEET concentrations in surface

water, with higher concentrations occurring during low flow conditions. However, their study involved monitoring a sand trickling filter WWTP which was less efficient in removal of DEET compared to the activated sludge method (Aronson et al, 2012). Therefore, much higher concentrations of DEET were present in receiving water in the said study and thus concentration fluctuations were more apparent (0.3 – 15 µg/L compared to average of 50 ng/L in the current study). Given the relatively low loadings to the receiving water in this study, it was possible that the effects of seasonal condition changes were not significant enough to be detected (Figure 3-4a).

Other PPCPs had demonstrated different seasonal occurrence trends. Triclosan and trimethoprim are antimicrobials found in daily personal hygiene products, so their usage and concentrations in wastewater influents should be relatively independent of season. However, the greatest concentrations in surface water were observed in March samples while other samples from August, September and January had similarly lower concentrations (Figure 3-4b). Triclosan is susceptible to photo-degradation and therefore is usually found at low concentrations in summer when daytime is prolonged (Lindstrom et al, 2002). During the winter (January), a proportion of these chemicals may have accumulated in ice and snow. It was also possible these compounds settle in sediments during low light and low flow conditions in winter. During snowmelt in early spring (March), more of these sequestered compounds may be released into surface waters and thus a concentration spike may be observed. Trimethoprim shares similar chemical structures and chemical properties as triclosan and would also demonstrate similar seasonal patterns of occurrence in surface waters. Overall, early spring during snow melt appeared to be when concentrations of various chemicals spike up.

3.5 Conclusions

The City of Swift Current WWTP releases generally low but significant amounts of chemical contaminants into Swift Current Creek. The activated sludge method has proven high removal efficiency for most of the organic compounds that were examined and illustrated in this study. However, a significant number of chemicals were persistent in the treated effluent because they either were not efficiently removed by this treatment method or they were present in very high concentrations in raw sewage. The residual proportion of chemicals from this treatment method could still result in significantly high concentrations of contaminants when the influent contains a heavy load of chemicals.

The most concerning chemicals in the aquatic environment are natural and synthetic hormones suspected to have endocrine disrupting effects. From this study, they appeared to be efficiently treated by the WWTP. However, previous studies have shown that hormones and their mimics could cause adverse effects in aquatic environments even at very low concentrations. Our results have also suggested that the WWTP is not the only source responsible for hormone inputs, as livestock from nearby farms had also evidently contributed to the composition in the creek.

As expected, the WWTP effluents were not a major contributor of pesticides. Higher concentrations of pesticides were found in surface water compared to WWTP influents and effluents, which further supported the hypothesis of the absence of pesticides in municipal sewage. This observation should also bring attention to pesticide applications in nearby farmlands. Previously, many pesticides have been banned once proven to have negative impacts and thus their route of exposure to aquatic environment should not be overlooked. The observed incidental inputs of both hormones and pesticides indicates that other possible routes of entry

outside of obvious point source discharges into the aquatic environment to be considered when monitoring surface water.

PPCPs illustrated various behaviour in the creek. Due to the fact that most of their effects are unknown, it was difficult to conclude how the aquatic environment would be impacted. Triclosan, trimethoprim, and possibly other planar compounds illustrated increased concentrations in the spring, coinciding with the reproductive periods for many organisms. Therefore, it is warranted to monitor the occurrence of these compounds more closely, especially in the spring. In addition, toxicity tests for these compounds, and any other compounds that appeared at significantly higher concentrations in the creek should be conducted. It would present a clearer picture summarizing the potential environmental impacts that the WWTP effluent could have on the receiving surface water.

The Orbitrap allowed us to analyze multiple compounds in complex extracts like wastewater influents and effluents. This analytical method had some limitations, as detection of certain compounds in complex matrices can be difficult without extensive sample pre-treatment. Fractionation, sample cleanup and/or purification could reduce the complexity of the sample matrix, as well as isolate chemicals with similar properties. Each fraction could be more easily analyzed and targeted compounds can be more precisely quantified. Another promising aspect of the Orbitrap is that it has high resolution and can separate more than four hundred chemical compounds in a single wastewater sample (Robles-Molina et al, 2014). Even when not all standards are available, it would be possible to compile semi-quantitative data through non-targeted scanning to analyze other contaminants that are present. Regardless, this method can be very practical for the monitoring of surface waters for which sample matrices are less complex.

3.6 Tables and Figures

Table 3-1: Chemical standard used for Orbitrap analysis and sample spikes

Chemical Standard	Chemical Formula	Type	Supplier	CAS#
Ethinyl Estradiol	C ₂₀ H ₂₄ O ₂	Hormone	Cambridge Isotope	57-63-6
Estradiol	C ₁₈ H ₂₄ O ₂	Hormone	Cambridge Isotope	50-28-2
Estrone	C ₁₈ H ₂₂ O ₂	Hormone	Cambridge Isotope	53-16-7
Progesterone	C ₂₁ H ₃₀ O ₂	Hormone	Cambridge Isotope	57-83-0
Testosterone	C ₁₉ H ₂₈ O ₂	Hormone	Cambridge Isotope	58-22-0
Atrazine	C ₈ H ₁₄ ClN ₅	Pesticides	Sigma Aldrich	1912-24-9
Chlorpyrifos	C ₉ H ₁₁ C ₁₃ NO ₃ PS	Pesticides	Sigma Aldrich	2921-88-2
Pentachlorophenol	C ₆ HCl ₅ O	Pesticides	Sigma Aldrich	87-86-5
Triclocarban	C ₁₃ H ₉ C ₁₃ N ₂ O	Pesticides	Cambridge Isotope	101-20-2
Caffeine	C ₈ H ₁₀ N ₄ O ₂	PPCP	Cambridge Isotope	58-08-2
Carbamazepine	C ₁₅ H ₁₂ N ₂ O	PPCP	Sigma Aldrich	298-46-4
Clofibrate	C ₁₂ H ₁₅ ClO ₃	PPCP	Sigma Aldrich	637-07-0
DEET	C ₁₂ H ₁₇ NO	PPCP	Cambridge Isotope	132-62-3
Diazepam	C ₁₆ H ₁₃ ClN ₂ O	PPCP	Sigma Aldrich	439-14-5
Gemfibrozil	C ₁₅ H ₁₈ O ₂	PPCP	Cambridge Isotope	25812-30-0
Ibuprofen	C ₁₃ H ₁₈ O ₂	PPCP	Cambridge Isotope	15687-27-1
Miconazole nitrate	C ₁₈ H ₁₄ Cl ₄ N ₂ O	PPCP	Sigma Aldrich	22832-87-7
Naproxen	C ₁₄ H ₁₄ O ₃	PPCP	Cambridge Isotope	22204-53-1
Sulfamethoxazole	C ₁₀ H ₁₁ N ₃ O ₃ S	PPCP	Cambridge Isotope	723-46-6
Triclosan	C ₁₂ H ₇ Cl ₃ O ₂	PPCP	Cambridge Isotope	3380-34-5
Trimethoprim	C ₁₄ H ₁₈ N ₄ O ₃	PPCP	Sigma Aldrich	738-70-5

Table 3-2: Extraction recovery (%) of spiked water samples from three matrices of concern: wastewater influent, wastewater effluent and river water. Each matrix was spiked in triplicate, average results are shown (n=3)

Chemicals	Influents (%)		Effluents (%)		River (%)	
	Mean	SD	Mean	SD	Mean	SD
Atrazine	0.00	0.00	0.00	0.00	0.00	0.00
Caffeine	44.31	10.51	9.81	16.99	0.00	0.00
Carbamazepine	270.81	116.71	285.65	68.00	3.68	6.38
Chlorpyrifos	0.00	0.00	0.00	0.00	1.29	1.15
DEET	369.41	110.01	0.00	0.00	11.55	10.39
Diazepam	150.70	65.52	103.31	28.24	45.39	10.30
EE2	0.00	0.00	18.58	5.52	89.59	9.24
Miconazole Nitrate	0.00	0.00	14.57	1.12	14.02	2.91
Pentachlorophenol	79.48	26.93	108.23	13.78	114.17	24.04
Progesterone	17.51	16.28	70.04	5.68	82.13	11.79
Sulfamethoxazole	0.00	0.00	0.00	0.00	0.00	0.00
Testosterone	74.51	8.38	34.43	4.69	50.11	7.69
Triclocarban	63.23	27.68	38.81	20.02	16.15	15.71
Triclosan	48.63	14.83	70.23	3.34	100.85	5.77
Trimethoprim	59.74	15.46	35.92	9.58	7.22	1.62

Table 3-3: Pesticides detected in Swift Current WWTP and Swift Current Creek. Mean values were calculated from all four sampling dates. Non-detect samples are listed as less than detection limit.

Chemical	Matrix	Mean (ng/L)	Minimum (ng/L)	Maximum (ng/L)
Atrazine	Influent	2.12	0.32	3.81
	Effluent	0.96	<0.25	1.79
	Surface Water	1.71	<0.25	6.45
Chlorpyrifos	Influent	0.38	<0.025	1.28
	Effluent	0.41	0.03	0.88
	Surface Water	0.61	<0.025	1.16
Triclocarban	Influent	<0.25	-	-
	Effluent	<0.25	-	-
	Surface Water	<0.25	-	-

Table 3-4: Pharmaceuticals and Personal Care Products Detected in Swift Current WWTP and Swift Current Creek. Mean values were calculated from all four sampling dates. Non-detect samples were listed as less than detection limit. Extraction recovery shown in Table 2 were not taken into account.

Chemical	Matrix	Mean (ng/L)	Minimum (ng/L)	Maximum (ng/L)
Caffeine	Influent	8467.51	673.32	20673.15
	Effluent	2.04	0.79	4.71
	Surface Water	1.44	<0.25	3.06
Carbamazepine	Influent	71.73	2.13	216.43
	Effluent	13.65	0.59	46.21
	Surface Water	2.77	<0.025	10.90
Clofibrate	Influent	483.33	<0.25	1712.59
	Effluent	49.89	<0.25	100.10
	Surface Water	249.82	<0.25	724.07
DEET	Influent	1692.33	533.14	4068.31
	Effluent	29.67	4.45	87.11
	Surface Water	49.97	<0.025	212.28
Diazepam	Influent	<0.25	-	-
	Effluent	<0.25	-	-
	Surface Water	<0.25	-	-
Gemfibrozil	Influent	10063.51	<2.5	29592.44
	Effluent	541.42	<2.5	2083.84
	Surface Water	24.96	<2.5	223.87
Ibuprofen	Influent	<12.5	-	-
	Effluent	<12.5	-	-
	Surface Water	<12.5	-	-
Miconazole Nitrate	Influent	<0.025	-	-
	Effluent	<0.025	-	-
	Surface Water	<0.025	-	-
Naproxen	Influent	<12.5	-	-
	Effluent	<12.5	-	-
	Surface Water	<12.5	-	-
Sulfamethoxazole	Influent	<2.5	-	-
	Effluent	<2.5	-	-
	Surface Water	<2.5	-	-
Triclosan	Influent	595.96	72.26	1041.27
	Effluent	34.15	0.87	126.68
	Surface Water	5.83	<0.25	22.01
Trimethoprim	Influent	5.12	<0.25	9.03
	Effluent	3.18	<0.25	6.98
	Surface Water	6.31	<0.25	20.58

Table 3-5: Natural and Synthetic hormones detected in Swift Current WWTP and Swift Current Creek. Means were calculated from all four sampling dates. Non-detect samples were listed as less than detection limit. EE2, E2 and estrone, despite having low detection limits, have poor extraction recovery in complex matrices (wastewater influents and effluents). Thus values reported are considered to be lowest possible concentrations.

Chemical	Matrix	Mean (ng/L)	Minimum (ng/L)	Maximum (ng/L)
Ethinyl Estradiol	Influent	<0.025	-	-
	Effluent	0.81	<0.025	3.13
	Surface Water	0.50	<0.025	4.46
Estradiol	Influent	1.97	<0.25	7.89
	Effluent	0.38	<0.25	1.51
	Surface Water	0.55	<0.25	5.89
Estrone	Influent	9.04	<0.025	35.16
	Effluent	0.68	<0.025	2.70
	Surface Water	0.25	<0.025	2.27
Progesterone	Influent	<2.5	-	-
	Effluent	<2.5	-	-
	Surface Water	<2.5	-	-
Testosterone	Influent	<2.5	-	-
	Effluent	<2.5	-	-
	Surface Water	<2.5	-	-

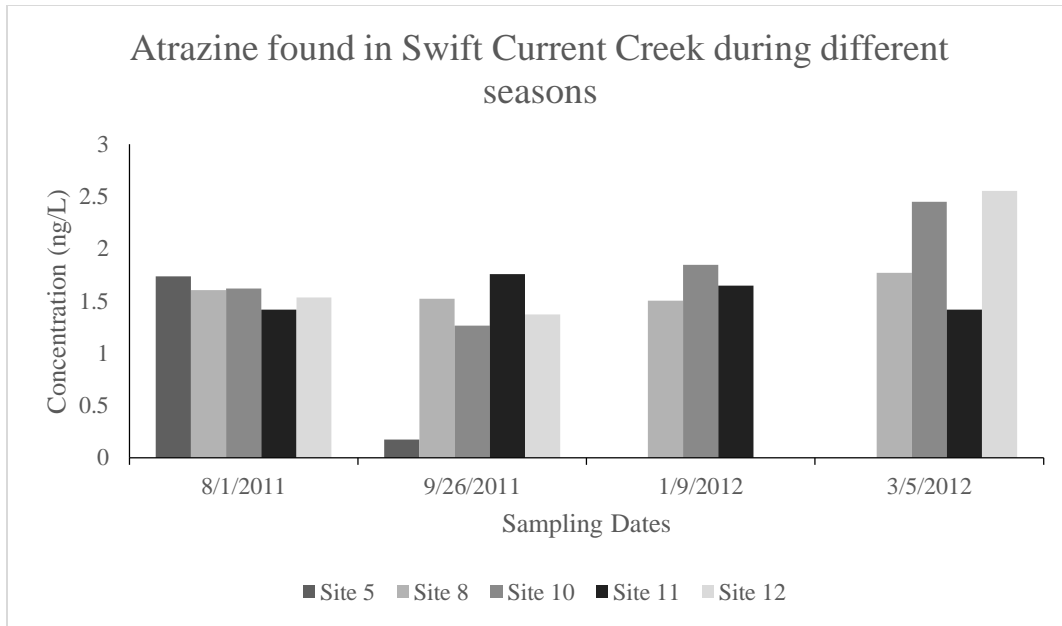


Figure 3-1: Atrazine found in Swift Current Creek downstream of the WWTP. No significant trend to suggest dilution effect, thus it was believed that atrazine was input consistently into Swift Current Creek via non-point sources.

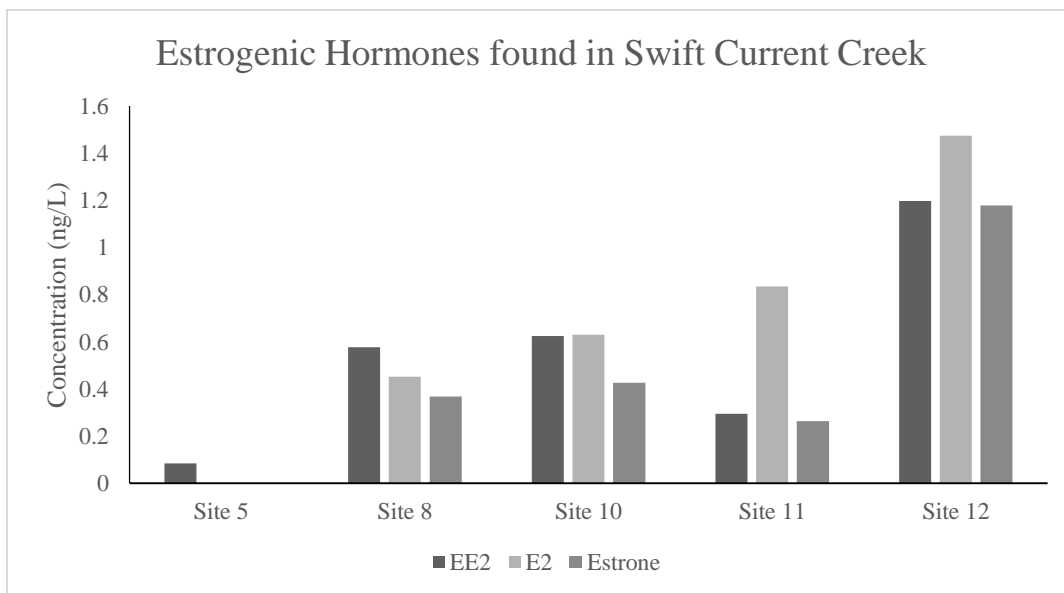


Figure 3-2: Average concentrations of estrogenic hormones detected in Swift Current Creek downstream of the WWTP from four sampling dates. Consistently low levels found in all sites with no trend suggesting dilution effects. Results indicating that low level of point discharge from the WWTP is rather persistent in surface water.

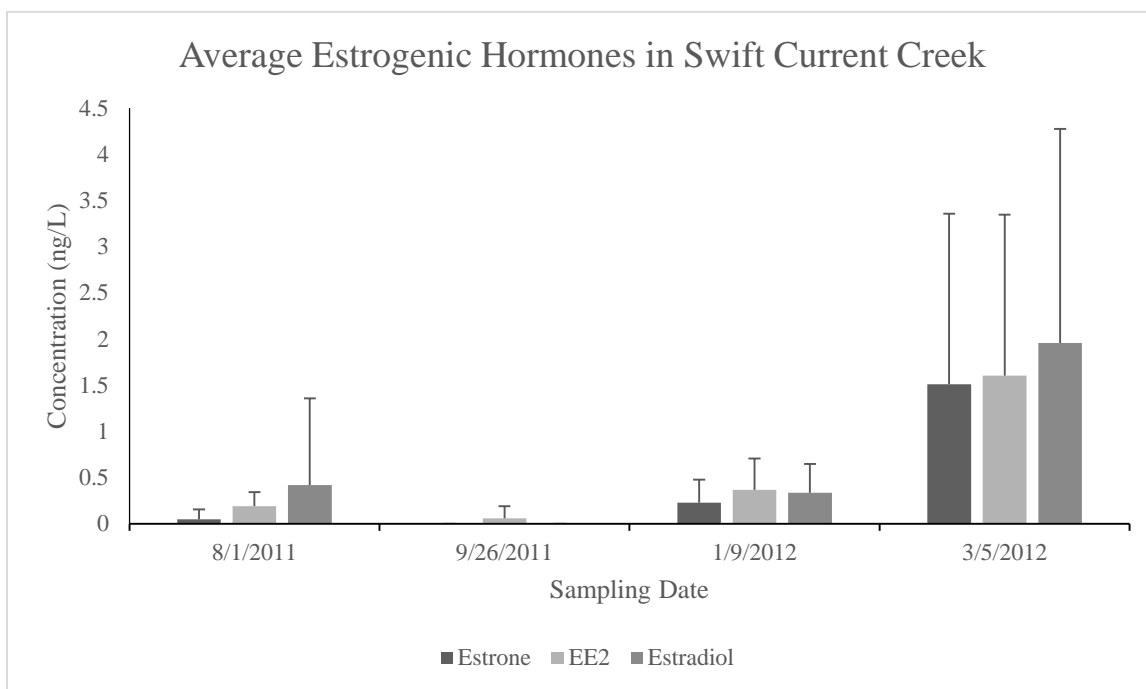


Figure 3-3: Average concentrations of estrogenic hormones in all downstream sites in Swift Current Creek. As shown, January and March, representing winter and spring (low flow seasons) had higher concentrations than August and September, representing summer and fall season (high flow seasons).

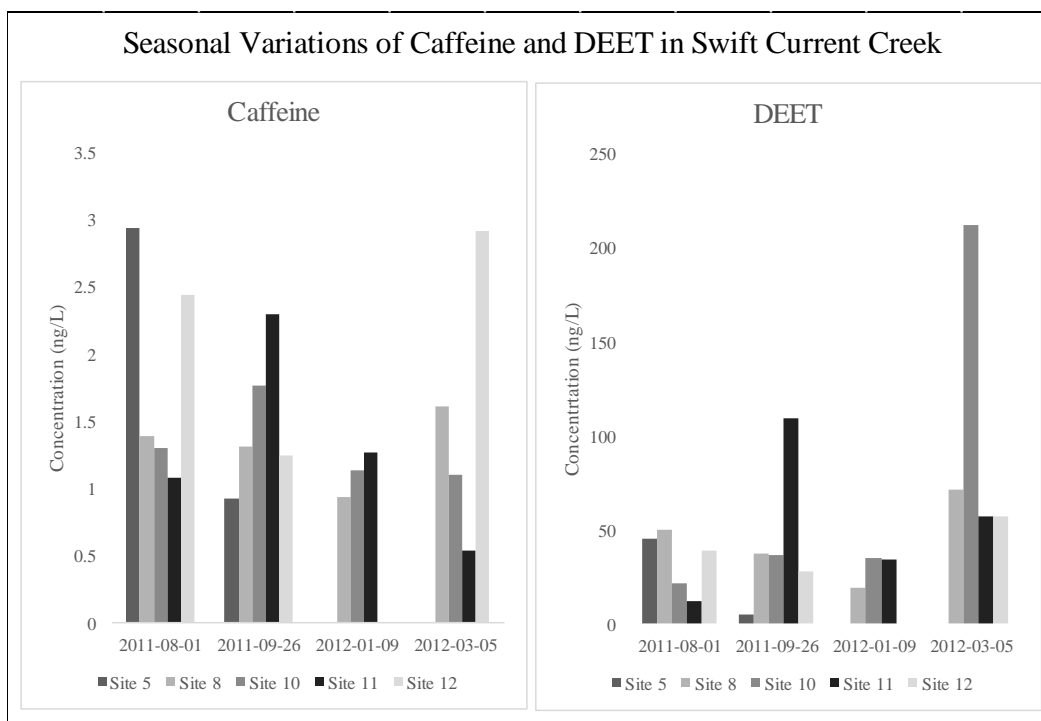


Figure 3-4a: Seasonal variation of Caffeine and DEET detected in Swift Current Creek. Concentrations of these two chemicals are rather consistent throughout the year due to great removal efficiencies by the WWTP and resulted in relatively low concentrations found in surface water.

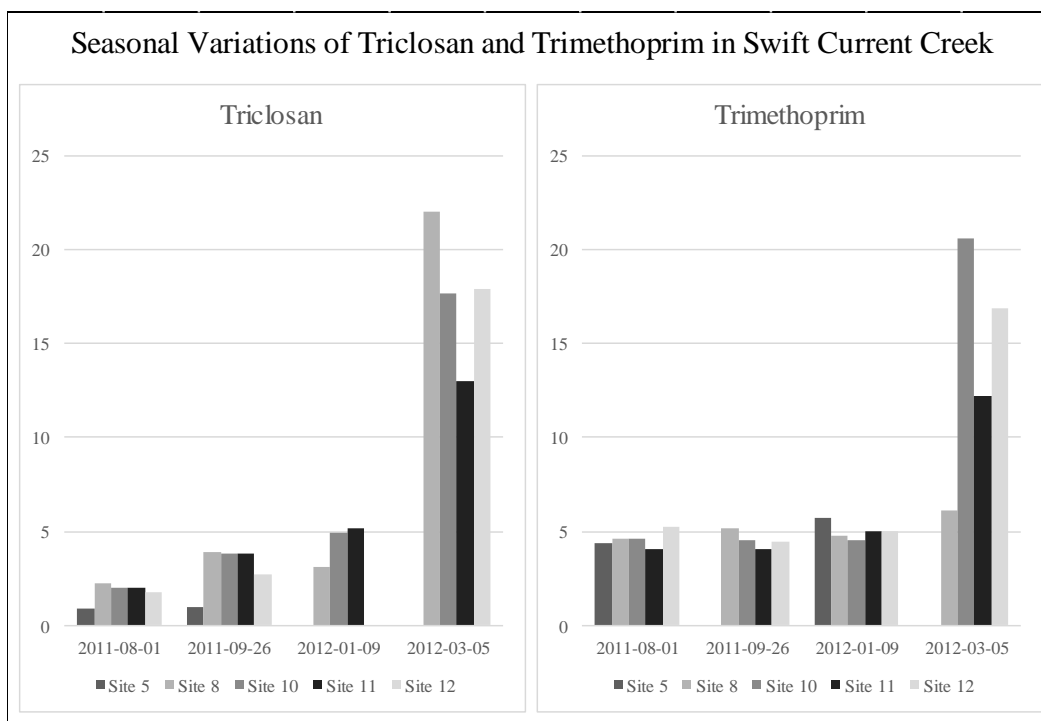


Figure 3-4b: Seasonal variation of Triclosan and Trimethoprim detected in Swift Current Creek. Peak concentration was observed in March which believed to be due to sequestered chemicals in ice released during snowmelt.

CHAPTER 4: Conclusions

4.1 Swift Current Creek

Pre-screening raw and treated water samples from the WWTP as well as water samples from Swift Current Creek downstream of the WWTP with a battery of cell bioassays revealed near baseline responses across treated water samples (effluents and lagoon samples) as well as surface waters. As expected, no responses were apparent in H4IIE cells, indicating the absence of PAH and dioxin-like chemicals, which are not usually found in municipal sewage. Similarly, androgenic activities were only detected in WWTP influent samples and not in effluents and downstream surface waters. The near-complete removal of testosterone and its agonists agreed well with previous studies (Liu et al, 2009; Leusch et al, 2006). Although minimal, estrogenic activities were observed in treated effluents and surface water. The apparent absence of estrogenic activities in influent samples raised concerns about our analytical method, and thus led us to investigate further by analyzing samples using the Orbitrap.

EEQs were calculated for each sample using EEQ factors listed in previous studies and the concentrations of estrogenic hormones detected in our samples by the Orbitrap. For comparison, statistical analysis was used to determine the threshold EEQ to elicit a response above baseline in the MVLN cell assay. The majority of samples had EEQ values well below the baseline threshold of the MVLN assay, thus responses above the baseline could not be detected. Furthermore, the influent samples, due to complex matrix effects, caused cytotoxicity when dosed at higher concentrations. Instrumental analysis was also challenging with the influent samples as the matrix presented high noise and interference and extract clean-up would be required in order to accurately assess these samples. In summary, based on results that we have obtained from the cell bioassay test, we conclude that the treatment process implemented at the

Swift Current WWTP removes nearly 100% of the hormone activity. As a result, receiving surface water (i.e. Swift Current Creek) was not impacted by the hormones discharged by the WWTP, as only baseline activities were seen in downstream samples.

The second part of this study was to construct a chemical profile in Swift Current Creek by analyzing the field samples more closely using instrumental techniques. Using an Orbitrap Hi-Res HPLC/MS system, we were able to identify and semi-quantify a range of chemicals. In addition to PPCPs, we also targeted a few pesticides (atrazine and chlorpyrifos). Although pesticides are generally not expected to be found in municipal wastewater, Swift Current Creek is in close proximity to farmlands as so may be impacted by leaching and/or runoff of agricultural contaminants. Pesticides were detected at low concentrations in wastewater but had elevated concentrations in surface waters. Due to low concentrations in WWTP samples, combined with the general lack of a spatial trend demonstrating any dilution effects, we concluded that the levels of pesticides (mainly atrazine) detected in the creek were mainly attributed to runoff from nearby farmlands (i.e. non-point sources). Seasonal variations in atrazine concentrations were minimal. A possible explanation was that the projected seasonal variation in the amount of atrazine was masked by the river flow of Swift Current Creek. During summer when atrazine was widely applied, the flow rate of the creek was also high and thus flushing and diluting the amount of atrazine that ran off into the water. In contrast, during late fall and winter when pesticide applications were absent, the flow rate was also lower.

Upon closer examination of the hormone data obtained Orbitrap analysis for Swift Current Creek, we observed low yet significant amount of estrogenic hormone present in treated sewage. The levels detected by the Orbitrap was much lower than the detection limit by MVLN cell assay. Therefore, based on the Orbitrap results, it was concluded that the removal of

estrogenic hormones by the WWTP treatment was incomplete. Furthermore, when comparing effluent samples and lagoon samples, the level of estrogenic hormones detected between the two groups of treated wastewater samples were very similar. This observation suggested that neither activated sludge nor passive removal using lagoons can completely eliminate estrogenic hormones and their mimics from municipal wastewater. Results from the lagoon samples also indicated that a portion of estrogenic hormones do not bind to particulates nor biodegrade overtime in the lagoon. Therefore, these compounds would also be bioavailable to aquatic organisms when released into the downstream surface water. The low ng/L range of concentrations of these hormone mimics detected in surface waters were similar to concentrations associated with chronic adverse effects in aquatic organisms reported in previous studies (Purdom et al, 1994; Jobling et al, 1998; Allen et al, 1999; Johnson and Sumpter, 2001; Segner et al, 2003).

Both temporal and longitudinal variations of hormones in Swift Current Creek were evident. Due to consistent level of input of hormones from the WWTP year-round, estrogenic hormone concentrations in Swift Current Creek are largely influence by seasonal flow rate. In addition, March had higher concentrations than January, suggesting hormones may have been trapped in snow and ice during winter and released back into the water during snowmelt. This hypothesis raises further concerns as the observed phenomenon coincides with the spawning season of many aquatic organism. Couple of studies in China also observed similar temporal trend and suggested that it could also be due to lower efficiencies of WWTPs in winter, thus resulting in higher concentrations in early spring (Sui et al, 2011; Yu et al, 2013). Along the creek downstream of the WWTP, the farthest sampling point from the WWTP, Site 12, had higher estrogenic hormone concentrations detected than other sites. This indicates that discharge

sources other than the WWTP may be present. One observation made was the presence of livestock near the surface water, which could have contributed to higher hormone levels.

Pharmaceuticals and personal care products presented more variable trends in this study compared to the other two groups of contaminants examined. Some chemicals that we examined were very efficiently removed by the WWTP treatment. Caffeine and DEET, each with thousands of ng/L input into the sewage, demonstrated on average >98% removal. As a result, these chemicals, due to their consistently low presence in downstream surface water, showed minimal seasonal or longitudinal variations and do not pose a threat to the aquatic environment. In contrast to these chemicals, many pharmaceuticals appeared to be more persistent in municipal sewage. In this study, carbamazepine, clofibrate and trimethoprim exhibited similar concentrations in both influents and effluents. On the contrary, gemfibrozil illustrated a relatively high removal efficiency (84%). However due to its high input, the residual proportion still yielded relatively great concentrations (hundreds of ng/L) in effluents and lagoon samples. For many of these chemicals, their aquatic toxicity and their potential impact to the aquatic environment remain unknown, making it difficult to assess potential risks.

Similar to hormones, an early spring spike in concentrations was observed among certain PPCP species. Triclosan and trimethoprim are antimicrobials found in many toothpaste and other daily cleaning products. Their usage is relatively consistent and occurrence in sewage should not be season dependent. However, we measured much higher concentrations of triclosan and trimethoprim in our March samples than other samples tested. Previous studies have stated that planar chemicals like triclosan and trimethoprim have tendencies for photodegradation and accumulation in ice and snow. Based on this theory, during summer and fall when the days are longer, these chemicals were more exposed to light and breakdown faster. During winter, they

will be drawn out of the liquid phase and accumulate in ice. During snowmelt in spring, large amounts of these types of chemicals were once again released back into the water and thus cause a spike in concentrations. Hence, it is important to assess toxicity of triclosan, trimethoprim, and other chemicals that share similar chemical structures and properties that can facilitate this spring time phenomenon. Overall, the Swift Current WWTP was able to remove the majority of the chemicals in raw sewage efficiently. Nevertheless, certain appear to exceed the capacity of the current WWTP system had been exceeded.

4.2 Effect-Directed Analysis and Future Research

As proposed, effect-directed analysis was able to provide us a snapshot of the toxicity profile of each type of sample collected. Dioxins/furans as well as other polycyclic aromatic hydrocarbons are often identified as carcinogens and pose potential adverse effects to aquatic organisms even at very low concentrations. The H4IIE cell assay is a very robust test that we used to confirm the absence of dioxin-like activities in our water samples. The cell-line was simple to maintain for more than 10 generations and 24-hour incubation was sufficient to induce quality responses. Although the H4IIE assay seems to have minimal applications when assessing municipal sewages, it could be very useful to quickly and regularly assess industrial sewage where dioxins and PAHs are expected to be more abundant.

Similarly and more importantly, the MDA cell bioassay was proven to be a viable option to assess municipal wastewater and monitor receiving surface waters when the effects of natural and synthetic androgenic hormones are under investigation. Using cell bioassays also has many advantages compared to bioassays using whole organisms. First, from an ethical point of view, using cell bioassays reduces the sacrifice of living organisms and thus is also more cost-efficient.

Secondly, cell bioassays generally have higher sensitivities compare to fish or other whole-organism bioassays. However, as seen in this study with MVLN assay, high sensitivity leads to additional challenges when the incubating environment compromises the baseline response. As proposed in Chapter 2, a novel T47D cell line is a more sensitive and promising option to assess estrogenic activities in water samples. This bioassay has a sensitivity that is approximately 10 times higher than the MVLN assay. However, in-house purification of the growth serum is required to strip any excess amount of hormones present in the culture media.

Modifications can be implemented in the EDA methods used in this study to provide more dimensions to surface water assessment. First, pre-filtered but un-extracted whole influent, effluent, lagoon and river samples could be used to directly dose each cell-line. Some previous studies have had success inducing dioxin-like responses or simple cytotoxicity responses using whole water samples (Dayeh et al, 2002; Shirmer et al, 2004). Slight variations in methods would have to be applied and validated. For example, the aforementioned studies made up growth media using either tissue culture water or whole surface and ground water. For the purposes of our study, we would maintain our cells in regular growth media and only expose the cells to whole water samples, sterilized and with media supplements, for the duration of dosing period (24 hours or 48 hours) and measure the cellular response post-exposure. One of the advantages of testing whole water samples is that it provides an even quicker and more cost-efficient method to obtain a preliminary evaluation of the samples. Another advantage is that by eliminating extraction and purification procedures in preparing the sample extracts, we are able to retain 100% of the total composition in each sample. Thus the results would be more realistic and relevant to the aquatic environment of interest. Last but not least, easy sample manipulation allows us to mix effluent samples and upstream surface water samples to simulate and predict

dilution effects when wastewater is discharged into the receiving water body. One potential challenge with this approach is that whole water samples may not provide a viable growth environment to the cells, especially for an extended incubation period. In addition, microorganisms present in the whole water could also present challenges. Handling and maintaining the cell lines can become difficult as cell cultures may have more opportunities to be exposed to contamination.

Field or laboratory whole-organism bioassays could also be implemented in future intended effect-directed analysis. Although it may not be practical for regular monitoring protocols, whole-organism bioassays are useful to study unknown adverse effects of novel chemicals. Biological responses elicited in whole organisms can be a more practical and relevant assessment of potential risks and effects in the aquatic environment. Previous studies have implemented these bioassays in the field and in the laboratory as effect-directed analysis (Goudreau et al, 1993; Rostkowski et al, 2011). Despite much lower sensitivity in these assays compared to cell bioassays, whole-organism assays allow us to monitor complex endocrine processes that are difficult to simulate on a cellular level (Rostkowski et al, 2011), as well as monitor changes in behaviours in addition to biological changes (Goudreau et al, 1993). However, these bioassays can also be much more costly than cell bioassays, especially when extended periods of exposure are used to assess chronic effects.

Preliminary experiments using Orbitrap LC-MS yielded promising results in this study. The total ion scan combined with more precise ion mass accuracy allowed the separation of hundreds of chemicals in a single sample. This is particularly useful for the analysis of highly complex samples with largely unknown matrices like wastewater influents and effluents. In addition to MS scans, targeted single ion monitoring (SIM) scanning enabled us to detect

specific compounds that we want to examine during water monitoring programs, despite high level of background noises. Modifications and improvements are certainly applicable to the current method to obtain better quality results. Sample clean-up and purification is most certainly required when analyzing raw wastewater samples in order to reduce noise and lower detection limits. The current method is semi-quantitative as surrogate standards were not spiked into each sample prior to filtration and extraction. This was due to the need for the sample extract to be used for both cell bioassay and instrumental analysis and we needed to prevent surrogate compounds from interfering with cellular responses elicited by targeted chemical groups. As a result, extraction recoveries of our samples cannot be accurately calculated. We should examine and identify suitable chemicals that are not our target and do not interfere with the cell assays (cells dosed with these chemicals in solution illustrate baseline response) and use them as surrogates for extraction and recovery efficiency. Last but most importantly, fractionation steps need to be implemented in order to isolate effect-causing agents as well as produce improved detections for real world samples. However, the additional labour and budget would need to be justified by the scope of the project.

4.3 Wastewater Monitoring Program

The City of Swift Current has a population of only 15,000 people as reported in 2011. For such a small city like Swift Current, the current WWTP's treatment method is sufficient to remove most of chemicals of concern, with minimal inputs to receiving surface water. However, previous studies (Clara et al, 2005; Carballa et al, 2004) and this study illustrated that certain chemicals are more resistant to current wastewater treatment methods. Therefore, wastewater monitoring should be more widely implemented geographically to provide updated information

regarding the chemical composition of wastewater effluents and receiving surface waters, especially in larger and more urbanized cities.

It was evident that concentrations of many chemicals were sharply elevated in the spring. This is potentially a serious concern as the spiking trend coincides with the spawning season of aquatic organism. The biggest obstacle that currently lies ahead is that the majority of PPCPs and other chemicals detected in wastewater have not been widely studied and their potential adverse effects are unknown. Consequently, it is difficult to establish a cost-effective procedure to monitor the specific chemicals of environmental concern. With the refinement of effect-directed analysis, it could be possible to carefully select for relevant targeted compounds. For example, since triclosan and trimethoprim were showing seasonal variations, the next step should be testing these two chemicals with the cell bioassays that were utilized in this study. If these two chemicals are indeed able to induce cellular responses at given concentration levels for any particular assay, a monitoring guideline can be set up.

This study has shown that other sources of pollutant discharges may also be present down the length of the creek. For example, livestock from nearby farms may be releasing natural hormones into the creek. In addition, for open-access surface waters like Swift Current Creek and Lake Diefenbaker, human recreational activities could also release potential contaminants into the water. Last but not least, pesticide applications during the farming season can lead to chemicals entering nearby surface water. Anthropogenic activities occur at the highest rate in summer and therefore demand us to monitor more closely during this period. One suggestion is to sample more frequently during summer to construct more detailed trends in contaminant concentrations and to increase the chance of capturing any abnormal and episodic activities that may result in sudden changes in surface water quality. Additionally, although concentrations in

summer may fluctuate the most, cell bioassays are generally not sensitive enough to detect these changes. However, some contaminants may have prolonged effects even at low concentrations if aquatic organisms are exposed during critical developmental stages (Nash et al, 2004; Lange et al, 2008). Therefore, instrumental analysis should be implemented more frequently for summer samples to achieve more accurate quantifications at low concentrations. For wastewater monitoring on site of the WWTP, cell bioassay is recommended over instrumental analysis due to simple methods as well as lower cost.

4.4 Conclusions

From this investigation, there was no clear answer to all of the studied hypotheses. The wastewater treatment plant removed most chemicals at a very efficient rate. However, some chemicals did show persistence as they were found at comparable levels in both the influent samples and effluent samples. On the other hand, chemicals like caffeine and DEET, which are widely used and present in very high concentrations in raw sewage, were effectively removed. Due to the high amount of input however, if even a very small proportion remains in the effluent, this could translate into a significant amount reaching the receiving water. No significant differences in chemical activities were observed between the WWTP treatment method and the conventional lagoon method, based on both EDA and instrumental analysis. This could be a source of concern as it may indicate that current treatment technologies are facing challenges in complete removal of certain chemical contaminants of concern, and these residual contaminants are not likely to bind to particulates nor biodegrade over time.

Hormones and some PPCPs were found at elevated concentrations in downstream sites. However, evidence also indicated that the WWTP was not the sole source of input for these

natural hormones and synthetic chemicals. In addition, agricultural leaching and runoff likely also contributed to pesticides found in Swift Current Creek. Seasonal variations were evident and the differences were due to natural environmental cycles (e.g. river flow rate) and chemical usage. The most obvious trend observed was that many chemicals had elevated concentrations in early spring during snow and ice melt. The significant amount of chemicals released into the water during spring thaw could affect the reproduction and development of aquatic organisms. Effect-directed analysis showed promising results and can be utilized for monitoring environmental impacts of wastewater.

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